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The technical objectives of this project were to: 1) transfect mutant p53s commonly mutated in human breast cancer into normal human mammary epithelial cells obtained from different donors and isolate clones; 2) characterize the clones for extension of lifespan and immortalization; 3) determine if expression of any of the mutant p53s provide a growth advantage to breast epithelial cells prior to immortalization; 4) determine in breast epithelial cells immortalized and expressing mutant p53 if expression of the mutant p53 is necessary for the maintenance of growth; and 5) determine downstream genomic targets of p53 that may be important in the development and progression of breast cancer.

The technical objectives accomplished include the determination of the effectiveness of different p53 mutants in extension of breast epithelial lifespan and immortalization; the spontaneous immortalization of Li-Fraumeni breast epithelial cells; the determination of the effectiveness of different promoters for achieving extension of lifespan and immortalization; and the determination of the role of telomerase in the development of human breast cancer.

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FOREWORD

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Jerry W. Shay 9/30/97

PI Signature Date

Table of Contents

The Role of p53 in Human Breast Cancer

Jerry W. Shay, Ph.D., Program Director

Lauren S. Gollahon, Ph.D, Postdoctoral Fellow

(Total grant period September 1, 1994 - August 31, 1997)

(Progress report period: September 1, 1996 - August 31, 1997)

Statement of Work:

The principal objectives are to:

1) transfect p53 mutants commonly observed in human breast cancer into normal human mammary epithelial cells obtained from different donors and isolate clones;

2) characterize the clones for extension of lifespan and immortalization;

3) determine in breast epithelial cells immortalized and expressing mutant p53 if expression of the mutant p53 is necessary for the maintenance of growth;

4) determine if expression of any of the p53 mutants provide a selective growth advantage to breast epithelial cells prior to immortalization;

5) determine downstream genomic targets of p53 that may be important in the development and progression of breast cancer.

Body:

The form that this report will reflect is as follows:

1) the technical concerns noted from the second annual report will be addressed;

2) relevant data collected directly pertaining to the statement of work will be presented;

3) significant new data stemming from ideas generated by findings from the original work indirectly involved with the grant specifications will be discussed.

4) Possible areas for future funding.

Introduction

Cellular immortalization has been classically thought of as the capacity for normal diploid cells to overcome cellular senescence. There appears to be two discrete stages, mortality stage 1 (M1) and mortality stage 2 (M2), that need to be overcome for human cells to bypass normal senescence programming before immortalizing. Both normal human fibroblasts and epithelial cells maintained in cell culture undergo cellular senescence and do not spontaneously immortalize. This model involving a two stage mechanism for regulating cellular senescence or aging has been previously reported (Wright *et al* 1989; Wright & Shay, 1992). In this model, the first stage or mortality stage 1 (M1) leads to normal cellular senescence. In order for cells to grow past this stage, the M1 mechanism must be overcome. This may be accomplished by interaction of mutant p53 with activated oncogenes such as Ha-ras (Eliyahu *et al* 1984) or by the activity of DNA tumor virus gene products such as human papilloma virus type 16 E6/E7; adenovirus 5 E1A/E1B or SV40 large T antigen (Demers *et al* 1994; Huang *et al* 1988; Linzer & Levine, 1979; Sarnow *et al* 1982; Shay *et al* 1991a; Shay *et al* 1993a; Shay *et al* 1993b; Werness *et al* 1990), presumably by inactivation of the tumor suppressor gene products pRb and p53. However, these DNA virus proteins fail to directly immortalize human cells. Cells overcoming M1 continue to proliferate in an "extended lifespan" period until an independent second mortality stage (M2) mechanism is activated (crisis), and only if a critical M2 gene becomes inactivated can a rare cell escape crisis and become immortal.

The p53 tumor suppressor gene product and the retinoblastoma gene product (pRb) or a retinoblastoma-like activity appear to be important in regulating the M1 stage in most human cell types(Hara *et al* 1996; Shay *et al* 1991b; Shay *et al* 1993c). In normal mammary epithelial cells the p53 levels remain constant throughout their lifespan in culture (this paper) in contrast to human diploid fibroblasts which showed an increase in levels of p53 as cells reached senescence (Kulju and Lehman, 1995). The pRb levels in HME cells, decrease significantly as the cells approach senescence (this paper). The p53 tumor suppressor gene is one of the most commonly mutated

genes in human cancer (Harris, 1996) with approximately 50% of primary breast tumors containing alterations involving the p53 gene (Callahan and Campbell, 1989; Hollstein *et al* 1991; Harris & Hollstein, 1992; Moll *et al* 1992). Cells may be immortalized through the introduction of viral oncogenes such as SV40 large T-antigen, adenovirus E1A/E1B, mutant p53, various human oncogenes such as c-myc, *H-* ras, and rarely, spontaneously. Prompted by the findings of Band *et al* (1990, 1991), that human mammary epithelial cells may occasionally immortalize when expressing HPV 16 or HPV 18 plasmids defective in pRb binding but normal for the E6 function of p53 abrogation, we infected human mammary epithelial cells (HME) with defective retroviruses expressing HPV 16 E6, E7 or E6/E7 (Shay *et al* 1993b). The results showed that HME cells expressing either HPV 16 E6/E7 or E6 alone were capable of overcoming M1 and in some instances M2. Direct support for this was recently obtained when it was demonstrated that breast epithelial cells but not breast stromal cells obtained from a patient with Li-Fraumeni Syndrome (containing a germline mutation in p53) spontaneously immortalized in cell culture(Shay *et al* 1995). That breast epithelial cells immortalize more easily than stromal cells is also supported by the epidemiological findings that the annual incidence of epithelial cell carcinomas such as breast cancer, are at least 100 times higher than the annual incidence of soft tissue sarcomas (Pollock, 1992). Tissue specific lineages are likely to have different regulatory mechanisms which may influence their ease of escape from senescence followed by immortalization. Although this does not necessarily indicate that bypassing M1 is less frequent in fibroblasts as studies such as Bond *et al* (1994) has been shown to successfully abrogate p53 function in human diploid fibroblasts with the introduction of mutant 53 143^{ala}. This is reflected in the apparently different roles that p53 and pRb have in cellular senescence for human mammary epithelial (HME) cells versus human mammary stromal (HMS) cells (Shay *et al* 1993b). The requirements to overcome the M1/M2 cellular senescence program appear to be more stringently regulated in fibroblasts than in epithelial cells.

Inactivation of M2 most likely involves recessive events consistent with the observations that a limited proliferative capacity is restored in hybrids between immortal and mortal cells (Pereira-

Smith & Smith, 1988; Shay *et al* 1993b). Mutational inactivation of one allele followed by the elimination of the remaining wild type allele by nondisjunctional or chromosomal conversions (Rew, 1994), selective growth advantage for the mutant (Harvey *et al* 1995) or possibly telomere shortening as theorized by Wynford-Thomas *et al* (1995), are all likely mechanisms for escape from M2. However, the gene product regulating the M2 stage still remains to be determined. One possibility is that it may involve a gene in the telomerase repression pathway (Shay and Werbin, 1993). The ribonucleoprotein enzyme telomerase, is involved in maintaining the stability of telomeres at the ends of chromosomes (Counter *et al* 1992). The end replication problem described by Watson (1972) would lead to progressive telomere shortening in normal cells since the mechanisms of DNA replication in linear chromosomes is different for each of the two strands (e.g., leading and lagging). This progressive loss of telomeres (simple tandem repeats of the sequence TTAGGG) at the ends of human chromosomes may be a molecular mechanism that determines the time of onset of cellular senescence (Olovnikoff, 1973; Harley, 1991).

Telomerase, an enzyme expressed in germ line cells, stem cells and cancer cells (Greider and Blackburn, 1985; Greider and Blackburn, 1989; Morin, 1989; Counter *et al* 1994; Kim *et al* 1994; Hiyama *et al* 1995; Piatezsek *et al* 1995) contains its own RNA template and thus, extends the overhanging G-rich telomeric strand by direct polymerization of deoxynucleotides into tandem TTAGGG repeats. This extended G-rich strand is now used as the template for synthesizing the C-rich complementary strand. Telomerase therefore stabilizes the telomeric length in immortal and cancer cells by compensating for the end replication problem. The absence of telomerase in normal somatic cells results in loss of 50-200 bp/cell from telomeres per round of replication (Harley *et al* 1990; Allsopp *et al* 1992). Bypassing M1 does not reactivate telomerase and telomeres continue to shorten during the period of extended lifespan (Counter *et al* 1992; Shay *et al* 1993). The immortal cells that overcome M2 almost always re-express telomerase and are capable of maintaining stable telomere lengths (Counter *et al* 1992; Wright and Shay, 1992; Shay *et al* 1993). Escape from M2 may thus represent the abrogation in the repression pathway of telomerase

activity in somatic cells (Counter *et al* 1992; Wright and Shay, 1992; Shay *et al* 1993; Piatyszek, 1995).

In the present grant study year we sought to determine if the M1 mechanism in HME cells could be directly overcome by the introduction of p53 mutants implicated in a variety of human cancers. While some of the p53 mutants inserted into normal human mammary epithelial cells resulted in extension of *in vitro* lifespan, one clone expressing p53 mutant 273^{his} immortalized.

The ribonucleoprotein enzyme telomerase has been shown to be active in 85% of primary human tumors tested and in over 90% of tumor-derived and experimentally immortalized cell lines. The proposed function of telomerase is thought to be in the maintenance of telomeric length in immortal cells. Because telomeric repeats (TTAGGG) are lost with each successive cell division, this "end replication problem" is considered an important biological timing mechanism (clock) that may determine the replicative capacity of all somatic cells [for review see (Harley, 1991)]. The upregulation or reactivation of telomerase appears to correlate with unlimited proliferation potential of a cell, hence immortalization. Yet there are cell lines in which the reactivation of telomerase is not observed. Telomeres are also important in the maintenance of chromosomal stability. In cells that bypass the normal cellular senescence pathway [for review see (Harley *et al* 1994)], the reactivation of telomerase occurs when the telomeres have reached a critically shortened length. However, several examples of experimentally immortalized cultured cell lines exist that proliferate indefinitely and with long, heterogeneous telomere lengths, without the presence of detectable telomerase activity. Currently, there are no reports of telomerase-negative tumor-derived or immortal cell lines with short telomeres (<8 kb). The regulation of the alternative lengthening of telomeres (ALT) pathway has been postulated to be similar to the Rad 52 dependent recombination and telomeric fusion events observed in yeast. Telomere heterogeneity was originally described by for a single marked chromosome, where alternating telomere lengths were observed during continued culturing of a telomerase negative, immortal cell line. Bryan *et al* (1995) also described immortal fibroblasts with long and heterogeneous telomeres and no detectable telomerase activity. In the same study, Bryan *et al* (1995) made somatic cell hybrids between telomerase positive x

telomerase negative cell lines and showed that the resulting clones were either telomerase positive or negative with some of the positive clones senescing. Complementation studies of somatic cell hybrids have demonstrated that some immortal x immortal cell fusions may still senesce and that mortal x immortal hybrids senesce suggesting that mortality is a dominant trait . In addition, several studies of cellular senescence induced by microcell mediated fusion have been reported . One study demonstrated that the specific introduction of chromosome 3 into an immortal human renal cell carcinoma line expressing telomerase resulted in the downregulation of telomerase activity, progressive loss of telomere length, and eventual inhibition of cell growth .

In order to address the relationship of p53 status to telomerase activity, a study was conducted to demonstrate that the ability to re-activate telomerase or to utilize the ALT pathway to immortalization may be strain specific in fibroblasts derived from individuals with Li-Fraumeni Syndrome (LFS). Our results indicate that immortalization appears to be dependent on p53 status in LFS 087 (tel -) and LFS 041 (tel+) fibroblasts and in the LFS 041 cells, loss of functional p53 and telomerase reactivation (telomere length stabilization) appear to occur concurrently. However, spontaneous immortalization of telomerase negative LFS fibroblasts appears to remain dependent on the maintenance of telomere lengths greater than a preprogrammed, specific critical length.

Surveys from a variety of human cancers (for review see (Shay & Bacchetti, 1997) have consistently demonstrated the presence of telomerase activity. There have also been studies demonstrating the presence of telomerase activity in premalignant lesions as well as "normal" tissues adjacent to tumors, making telomerase an attractive addition to present pathology tests. While telomerase activity appears to correlate very well with malignancy, there are several areas that need to be addressed before this assay can be applied to a clinical setting. First, inhibitors of the PCR amplification step in the reaction could yield false-negative results. Although mixing experiments with telomerase positive extracts or inclusion of an internal standard have been shown to reduce the numbers of false negatives significantly. The result becomes null when PCR inhibitors are observed in a reaction. Thus, if there is telomerase activity in the sample, it would not be detected. Likewise, in diagnostic tests using bodily fluids (e.g. colonic effluents, bladder

washes, panceatic washes), PCR inhibitors are present, with the added caveat of limited quantities of material (i.e. fine needle aspiration) or small numbers of telomerase positive cells diluted in a background of normal telomerase negative cells or in a large volume of fluid.

In the next section, we report alternative methods for detection of telomerase activity in solid breast and colon tumor samples. We show that PCR inhibitors present in tumor samples are removed during an initial extraction, allowing detection of telomerase activity following a second or third extraction. We also show that telomerase activity may be detected in colonic effluents and fine needle aspirates from breast lesions by utilizing strept-avidin coated magnetic beads to bind telomerase elongation products and allow for PCR amplification of telomerase specific extension products.

Numerous reports have documented telomerase activity in a high percentage of a variety of tumors including those of the breast (Hiyama *et al* 1996), lung (Hiyama *et al* 1995), colon (Chadeneau *et al*, 1995; Tahara *et al* 1996), kidney (Mehle *et al* 1996), ovaries (Counter *et al* 1994), liver Tahara *et al* 1995), brain (Langford, *et al* 1995) and prostate (Sommerfeld, *et al* 1995), while in cleared margins around tumors telomerase activity is only rarely detected (Shay and Wright, 1996). Telomerase activity is absent in adult tissues, with the exception of male germline and embyronal cells (Wright *et al* 1996), proliferating cells of renewal tissues (Counter *et al* 1995; Broccoli *et al* 1996), basal epidermal cells (Harle-Bachor and Bouchamp, 1996; Taylor et el, 1996), intestinal crypt cells (Hiyama *et al* 1996) and activated lymphocytes (Shat *et al* 1996; Hiyama *et al* 1995).

These findings have stimulated much discussion and investigation of the potential diagnostic, prognostic and therapeutic implications for telomerase or telomerase antagonists in the management of cancer. In this study, we sought to determine whether telomerase activity can be detected in breast neoplasms using standard fine needle aspiration (FNA) techniques. We examined both benign and malignant breast masses to evaluate the potential diagnostic use of FNA-TRAP for breast masses. Our data indicate that telomerase activity can be reliably assessed by FNA-TRAP. Further, FNA-based TRAP is a highly sensitive test for malignant breast lesions.

Fibroadenoma of the breast is considered a benign disease of the stromal component of mammary glands. The ductal epithelium becomes compressed and irregular morphologically. Generally, the lesion regresses or at the request of the patient, is resected. Extensive review of the literature suggests that there may be a subset of women who present with FA that may be at greater risk for developing sporadic cancer later. Prior studies have shown that approximately half of FA assayed for the presence of telomerase activity were positive. The purpose of this study was to investigate the existence of a correlation between telomerase activity, telomerase RNA expression and known breast cancer markers in order to identify a subset of women with a genetic propensity for breast cancer

1) The first portion of this review is to address the summary review concerns pointed out from the second annual report.

A). Format/Editorial Issues Addressed:

Figure 7A has been reconfigured so that the different bars (and the trend) may be more easily distinguished. (Please See Figures and Figure legends).

B). Technical Issues Addressed:

a). In reference to page 6, part A and Figure 1: The population doubling levels (PDL) were determined by the following formula:

cells counted / # cell plated = X

log [X] / log [2] = population doubling of cells since last plating.

This value is then added to the PDL calculated before the last plating.

A Coulter™ counter was utilized for the most accurate possible counting procedures. These cells were initially cultured from primary tissues samples by our lab. Therefore, we were able to establish PDL from the initial time the cells were placed in culture. In reference to the question of

accuracy of these counts, it is our opinion that they are accurate and believable for the following reasons:

1). The cell cultures were initiated in our lab.

2). The use of the Coulter counter makes counting more accurate and convenient. Dilutional effects and poor resuspension are minimized due to the speed of counting. The error of the counts is generally less than 10% between samples.

3). Applied methodology for counting is repeated rotinely on all cell strains and lines in our lab, therefore it is an established laboratory technique which we have standardized for our use.

4). Concurrent control experiments using cultures incubated with BrdU have shown similar turn-over and doubling times for 5 of the cell lines/strains used. They are HME 31; HME 32; HME32(273)-1, HME 50-5 (precrisis and immortal) and H1299.

b). Page 6 and figure 2 onward:

Cell line	Origin	Mutant introduced	Method of introduction	Phenotype	Immediate Growth arrest at 32°C
H1299	Derived from a NSCLC*	Mock	Electroporation/lipofectin	Slow growing into a monolayer	None
LC-2	H1299	v143A	Lipofectin	Compact foci	yes
LL-1	H1299	v143A	Lipofectin	Slow growing into a monolayer	yes
LL-2	H1299	v143A	Lipofectin	Large, irregular	no
LL-3	H1299	v143A	Lipofectin	Fast growing - monolayer	no
EL-1	H1299	v143A	Electroporation	Slow growing monolayer	no
EL-2	H1299	v143A	Electroporation	Large, irregular	yes
EC-1	H1299	v143A	Electroporation	Compact foci, fast growing	no
EC-2	H1299	v143A	Electroporation	Compact foci, fast growing	no

NSCLC - non-small cell lung carcinoma. Three of the lines exhibited significant differences in growth rate at 32°C compared to the other lines. After 17 days in culture, the cells cease growing and remain sub-confluent in the dishes. All cell lines listed are independently, clonally derived.

2). In reference to pZipneo: this is a plasmid control for the experiment. This is a construct into which the p53 mutants were cloned. Its presence confirms the results of extension of lifespan being induced by the p53 mutants and not a consequence of the plasmid introduction into the target cells.

c). Page 7 and figure 6. The mutant used in the immunoprecipitation experiments was the v143a p53 mutant which is detectable using monoclonal antibody 240 (Oncogene Science). This antibody specifically recognizes the mutant conformation of the protein under non-denaturing conditions and binds. The wild type conformation may be detected with monoclonal antibody 1620 (Oncogene Science) in the same manner as the mutant. Both these antibodies will recognize any form of p53 in denaturing conditions. The progress of the conformational changes of this temperature sensitive mutant may then be tracked at the permissive 37°C and non-permissive 32°C temperatures. A polyclonal antibody specific for c-myc was used as a control.

d). In reference to the reviewer's question of normalization of the protein samples:
Each flask of cells to be extracted were lysed at 80% confluency. Thus, the cells were in log growth. All cells were lysed in the same buffer under the same conditions. The lysis buffer used consisted of 2% SDS, 5% sucrose and 0.05M tris. Cells were lysed on ice and the DNA was sheared by aspirating through a fine gauge needle. After shearing, a BCA protein assay (Pierce) was performed. 20μG each of protein was then denatured at 100°C for 5 min. in the presence of ß-mercaptoethanol and run on a 10% PAGE under denaturing conditions. The p53 antibody used was monoclonal DO-1 (Oncogene Science). These bands were nit quantified by scanning at the time. The larger areas most probably represent the relative expression of the protein rather than spread of band width as these gels were all run under the same conditions and transferred immediately onto nitrocellulose membrane. My interpretation of the results is that at 37°C only the introduced mutant p53 is present since H1299 cells are null for wild type p53 protein. At 32°C the conformational change to wild type may induce other p53 transcription factors to initiate an

upregulation in the production of the now wild type p53 and therefore not only might there be residual mutant protein, but also an increase in the relative amounts of wild type p53.

e). Page 8

1). HME 31 is a primary human mammary epithelial cell strain. The origin tissue was obtained from the adjacent normal tissue of a 53 yr old female undergoing radical mastectomy. This cell strain was first established in our lab.

HME 32 is also a primary human mammary epithelial cell strain. The origin of this tissue was a 23 yr old female undergoing reduction mammoplasty. This strain was also first established in our lab. Both strains were analyzed cytogenetically and at early PDL in culture both were normal, diploid XX. Senescing cells when analyzed however, showed karyotypic instability and aneuploidy.

2). Introductory sentence for section D.

Recent studies have shown that telomerase in stem cells may be induced to continue expression with the introduction of such viral oncogenes as HPV 16 E6 which targets p53 for degradation. If this is true, then this would show a direct relationship between p53 and telomerase activation. We have not observed a consistent direct correlation. In a number of similar experiments, our results have shown that it is the genetic variability of the strain, the PDL of the cell strain and the type of cell strain (i.e. cell lineage) which primarily appears to affect telomerase reactivation and p53 expression. Therefore in order to directly address these observations by Klingelhutz *et al* and Foster *et al* we conducted the following experiments.

Materials and Methods utilized in experiments pertaining to this grant study.

Cell lines and cell culture

Normal mammary epithelial cells (HME) 31 were obtained from a patient undergoing breast cancer surgery. HME 32 was obtained from a patient undergoing reduction mammoplasty. Cells were cultured as previously described (Van Der Haegen *et al* 1992). Briefly, serum-free medium was

utilized, consisting of a modified basal medium MCDB 170 (MEBM, Clonetics, San Diego, CA,) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA,); 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 25 μg/ml gentamicin, 10 ng/ml transferrin (Sigma, St. Louis, MO,); and 10 ng/ml epidermal growth factor, (Collaborative Research, Bedford, MA,). Medium was changed every other day. Primary cultures were originated from organoids and grew predominantly as epithelioid cell populations. Following a 'self-selection' process HME 31 could be subcultured for an additional 45 - 50 doublings while HME 32 could only be subcultureed for an additional 25 - 30 doublings. These cell strains have been previously characterized by our laboratory (Van Der Haegen *et al* 1993).

Li-Fraumeni cells: The cell lines studied (041 and 087) were derived from primary tissues obtained by skin biopsy from patients with Li-Fraumeni Syndrome. Characterization and immortalization of these cells *in vitro* was previously described . 041 cells contain a frameshift point deletion at codon 184 (GAT - GAA) which generates the translation of 60 aa from the resulting frameshift that are not normally in the p53 protein followed by a premature stop codon rendering the cell line null for p53 activity. 087 cells contain a missense point mutation at codon 248 (Arg - Trp). The growth and maintenance of these LFS fibroblasts was performed as previously described . All cells were grown in modified Eagle's medium with 10% fetal calf serum and antibiotics.

Other cell strains and lines: Human mammary epithelial (HME) cells derived from two different primary cultures were used. HME 50-5 cells are spontaneously immortalized cells originating from a patient with Li-Fraumeni Syndrome. HME 32(273)-1 is a p53 mutant 273 R(H immortalized cell line. Normal HME 73, 87, HMS 60, 67, 73 and 79 and 87 were also utilized. These cultures were maintained as previously described. Other cell lines assayed were H1299 (non small cell lung carcinoma) and clonal derivatives from a transduction experiment with p53 mutant v143a, HT1080 (fibrosarcoma), Hammon Camcer Center (HCC) tumor-derived breast cell lines 1187, 1419, 1500, 1569, 1739, 1806 and 1954. These mammary epithelial cell lines were grown

in RPMI (Gibco/BRL) containing 10% fetal calf serum (Hyclone Laboratories, UT) and 25 μg/mL gentamicin (Sigma).

Somatic Cell Hybrids.

Cells for preparation of somatic cell hybrids were harvested by trypsinization from rapidly growing cell cultures. Somatic cell hybrids were formed by mixing 3 X 10^6 cells of each pair HT1080(hygro^R) with 087ras(neo^R) or HT1080(hygro^R) with 041ras(neo^R), centrifuging at 800 rpm for 6 minutes, and washing once in 10 ml of serum free media, RPMI 1640. The cells were recentrifuged at 800 rpm for 6 minutes and resuspended in 1 ml of 50% polyethyleneglycol 1500 (Boehringer Mannheim) in RPMI 1640 prewarmed to 37(C. The cells were kept for 1 minute at 37(C in a water bath. After 1 minute, 1 ml of medium was added, and after 2 minutes, 2 ml of medium was added for a final volume of 8 mls. This mixture was kept for 4 minutes at 37(C, and then 4 ml of medium was added. The mixture was then centrifuged at 800 rpm for 6 min and resuspended gently in RPMI 1640. The washed cells were then centrifuged at 800 rpm for 6 min and resuspended in MEM with 10% FBS and plated at 1 X 10^6 cells per 100 mm tissue culture plate. Drug selection was initiated 24 h later in MEM with 10% FBS using both 100 mg/ml of hygromycin and 200 mg/ml G418. Double drug resistant colonies were picked 3-4 weeks later and used for further studies.

Tissue Samples

Forty-four female patients, age 14 to 82 (mean 45) and one male patient, underwent excisional biopsy of palpable breast masses. From these biopsies, 89 samples were obtained for telomerase analysis, including 46 fine-needle aspirates and 43 gross tissue samples. The FNA samples were obtained directly from the excised mass at the time of specimen removal in the operating room. The gross tissue samples were obtained immediately after the specimen was cut for frozen section. In some cases, FNA was obtained from more than one mass excised from the same patient. Additionally, gross tissue samples could not always be obtained for study due to small size of the

breast mass and thus, the possibility of interfering with histologic diagnosis in these smaller lesions.

Histologic Analysis

Invasive breast cancers were characterized using staging standards recognized by the American Joint Commission on Cancer. Rating scales for fibroadenomas were developed for four histologic categories: epithelial cellularity, stromal prominence, stromal cellularity and lymphocytic infiltration, and these rating scales are delineated below.

Histologic Rating Scale for Epithelial Cellularity:

Rating 1: very few epithelial cells - less than 10% of the cut surface occupied by epithelial cells; Rating 2: few epithelial cells - 10-20% of the cut surface epithelial cells; Rating 3: moderate epithelial cells - 20-30% of the cut surface epithelial cells; Rating 4: marked epithelial cells - greater than 30% of the cut surface epithelial cells.

Histologic Rating Scale for Stromal Prominence:

Rating 1: very little stroma - <30% of the cut surface occupied by stroma; Rating 2: mild stromal component - 30-40% of the cut surface stroma; Rating 3: moderate stromal component - 40-50% of the cut surface stroma; Rating 4: marked stromal component - >50% of the cut surface stroma.

Histologic Rating Scale for Stromal Cellularity:

Rating 1: very few stromal cells (hyalinized fibroadenoma) - < 10 stromal cells per 100X field; Rating 2: mild stromal cellularity - 10-50 stromal cells per 100X field; Rating 3: moderate stromal cellularity - 50-100 stromal cells per 100X field; Rating 4:marked stromal cellularity - > 100 cells per 100X field.

Histologic Rating Scale for Lymphocyte Infiltration:

Rating 1: rare lymphocyte in the cut surface of the specimen; Rating 2: between 1 and 10 small aggregates of lymphocytes seen in the cut surface of the specimen; Rating 3: many lymphocytes seen diffusely throughout the specimen, or more than 10 small aggegates seen in a cut surface of

the specimen, or a single germinal center seen in the cut surface of the specimen; Rating 4: many germinal centers seen in a single section of the specimen.

Fine Needle Aspirate (FNA) cell estimations

Each FNA extract sample (5 µl) was placed on a 100 mm petri dish containing 10 ml of a 0.08% agarose and 0.1 µg/ml of ethidium bromide (Sigma Chemical Co., St. Louis, MO) mixture. The samples were allowed to absorb into the agarose/EtBr for 10 min before visualizing with UV. Quantitation of the fluorescent signal was performed using an IS1000 Gel Documentation Imaging System (Alpha Innotech, Corp, San Leandro, CA). This quantity was then compared to the DNA constant for HeLa cells (15 pg/cell) (Darnell *et al*, 1986)) and a rough approximation was made based on the ratio of DNA concentration to cell number.

Recombinant retroviral vectors

Retroviral vectors consisted of the parent vector pZipNeoSV(X) or pZipNeoSV(X) containing the genes for 143^{ala} mutant p53; 175^{his} mutant p53; 248^{trp} mutant p53; 273^{his} mutant p53 under the transcriptional regulation of the Moloney murine leukemia virus promoter-enhancer sequences (MLVLTR). These vectors also contained the gene conferring neomycin resistance under the transcriptional regulation of the SV40 promoter. The mutant p53 vectors were all kindly provided by Curtis Harris (National Cancer Institute, Bethesda, MD). Recombinant viruses were generated in the amphotrophic packaging line PA317 according to previously described procedures (Shay *et al* 1993). Viruses produced from the PA317 cells were used to infect HME cell strains 31 at PDL 22 and 32 at PDL 18, similar to those described by Halbert *et al* (1991;1992). The cells were selected on G418 (50-100 µg/ml) and clones isolated. Each HME clone was subcultured and continuously passaged at 2 x 10^5 cells/T75 flask and tested for escape from crisis at a total population size of 1-2 x 10^6.

Transfections

HME 31 and 32 cells were Lipofectin (Gibco/BRL, Gaithersburg, MD) transfected at the same aforementioned PDLs with pRc/CMV constructs containing single p53 genes mutated at codons 143^{ala}, 175^{his}, 248^{trp} and 273^{his} respectively. These pCMV-Neo-Bam constructs were kindly provided by Dr. John Minna (Simmons Cancer Center, UT Southwestern Medical Center, Dallas, TX). The neomycin resistant gene was included as a dominant selectable marker. The transfections were performed under the following conditions optimized for HME cells; 10-20 µg of plasmid DNA was incubated with 150 -200 µl of Lipofectin at room temperature for 15-45 min. This newly formed complex was then added to 4 ml of Opti-MEM II transfection medium (Gibco/BRL, Gaithersburg, MD) and incubated at 37°C for 6-10 h. After incubation this medium was removed and replaced with fresh MEBM. Cells were allowed to recover for 24 - 48 h, then trypsinized, counted, replated at various densities with G418 added. After approximately 2 weeks,individual colonies were ring clone isolated and cultured as described above.

Transient Transactivation Analysis

Transient transfections were performed by electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA.) as previously described (Chen *et al* 1993). At 70-80% confluency HME 32 and HME 32(273)-1 cells approximately 2 $x10^7$ cells (0.5 ml) were mixed with 60 µg of DNA (20 µl) containing 10 µg pCMV-LacZ expression plasmid plus 10 µg of reporter plasmid supplemented with sonicated salmon sperm DNA. Cells were incubated at room temperature for 5 min followed by electroporation at 960 µF, 240 and 260 volts. Transfected cells were immediately re-suspended with pre-warmed medium and incubated under normal conditions. Within 12-24 hours, the medium was changed. Forty-eight to 72 hours post-transfection, cell extracts were prepared and luciferase activity measured as previously described (Funk *et al* 1992). Luciferase activity was assessed and normalized for differences in transfection efficiency as determined by a spectrophotometric ß-galactosidase assay

PCR amplification of pRc/CMV

The conditions for PCR amplification of sequences from within the CMV promoter of pRc/CMV are as follows: primers; 5'-ATAGTAATCAATTACGGGGTCATT-3', 5'-TATCGCTACTGATTATGCATCTAC-3'. These primers amplify a 347 base pair fragment which contains codons 260 607. Cocktail for the 50µl reaction contained 1.5mM $MgCl_2$, 2.5U of Taq polymerase (Gibco/BRL), 200mM dNTP's, 50pM of primers, 100 ng genomic DNA from HME 32 cells at PDL 18 or HME 32 (273)-1 cells at PDL 225, 20 ng plasmid DNA. PCR program was as follows: 94°C, 1 min; (94°C, 30s; 55°C, 30s; 72°C, 30s) x 40; 72°C, 1min. The PCR product was then run on an 0.8% agarose gel for 45 min. at 85V, stained with 0.05 µg/ml ethidium bromide and photographed.

Tetracycline inducible response promoter.

The TR-9 cells are LFS 041 cells that contain a repressible wt-p53 expression cassette. Stock cultures were maintained in 1mg/ml tetracycline, 600 mg/ml G418 and 50 mg/ml of hygromycin. For experiments, TR-9 cells were seeded at 2 X 10^6 per 100 mm^2 tissue culture plate in the presence of 600 mg/ml G418 and 50 mg/ml hygromycin. In cultures in which the wild type p53 was to be repressed, tetracycline was added to 1mg/ml. Cells were harvested at day 2, 4, 7, 9, 11 and 14 for cell counting, p53 expression and telomerase assays. The growth of the cells is almost immediately halted after removal of tetracycline from the medium. For the early times and for culture in which the wild type p53 was expressed, 2-3 plates of cells were harvested, pooled and counted.

Telomere length measurements

A. DNA from normal HME 32 and HME 32(273)-1 immortalized cells at PDLs 60, 80 and 100 was digested with Hinf1. 10 µg of each sample was run on a 0.8% agarose gel overnight at 70 volts constant. Gels were dried under vacuum at 50°C for 45 min, soaked in 0.5M sodium hydroxide/1.5M sodium chloride for 15 min, neutralized in 0.5M Tris pH 8.0/1.5M sodium

chloride for 15 min. Gels were then prehybridized in 5X SSC (standard saline/citrate), 5X Denhardt's, 0.5mM sodium pyrophosphate, 10mM disodium hydrogen phosphate at 37°C for 4-6 h. A second incubation in fresh solution with the ^{32}P end-labeled telomeric probe $(TTAGGG)_4$ for 12h followed. After washing three times in 0.1X SSC at room temperature (7 min each), the gel was either exposed to X-ray film or analyzed on a phosphorimaging device (PhosphorImager, Molecular Dynamics, Sunnyvale, CA, USA).

B. DNA from cultured cells was isolated by dialysis and digested with a six enzyme mix consisting of 10U each Hinf I, Alu I, Cfo I, Hae III, Msp I and Rsa I. 10µg of digested DNA was electrophoresed on 1.0% agarose gels using field inversion gel electrophoresis (FIGE) (Bio-Rad, Hercules, CA) with forward voltage 180V, reverse voltage 120V, for 20 hours. Gels were denatured in high salt buffer (0.5M NaOH, 1.5M NaCl) for 15 minutes, dried under vacuum at 50°C for 45 minutes and equilibrated in neutralization buffer (0.5M Tris-HCl, pH 8, 1.5M NaCl) 2 times for 15 minutes. The gels were preincubated in hybridization buffer (5X SSC, 5X Denhardt's solution, 0.5M sodium pyrophosphate, 10mM disodium hydrogen phosphate) at 37°C for 4 hours. Hybridization was done using fresh buffer and the radiolabelled telomeric probe $(TTAGGG)_4$ for 12 hours. Hybridized gels were washed 3 times for 7 minutes each with 0.1X SSC at room temperature and analyzed using the PhosphorImaging System from Molecular Dynamics. Terminal Restriction Fragment (TRF) lengths were estimated based on electrophoresis of 1kb and high molecular weight ladders (Gibco).

Western analysis.

Cells were harvested at approximately 80% confluence and analyzed as previously described. Cell extracts were prepared as per Gillespie and Hudspeth (1991), using a modified buffer lacking ß-mercaptoethanol until time of denaturation. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Immunoblotting, incubation and developing procedures followed the protocol for chemiluminescence detection of proteins as modified by Gillespie and Hudspeth (1991). Briefly, 20(g of protein extract was run under 10% SDS-PAGE conditions.

Resolved protein was transferred to ECL Nitrocellulose membrane (Amersham, Arlington Heights, IL) and incubated with a monoclonal, primary antibody [either anti-p53 clone Pab 1801 or DO-1 recognizing wild type and mutant conformations; (Oncogene Science, Cambridge, MA)], followed by a goat-antimouse IgG secondary antibody conjugated to alkaline phosphatase. pRb was detected using MAb-1 for the pRb gene product (Triton Diagnostics, Alameda, CA). Chemiluminescent reagents, nitro-block and CSPDL (Tropix, Bedford, MA), were used for signal detection and blots were exposed to Kodak X-ray film (Rochester, NY).

Telomerase assays

A one tube PCR-based telomerase assay was performed as originally described (Kim *et al* 1994) with some modifications (Wright *et al* 1995). The assay was performed in two steps: 1) Telomerase mediated extension of an oligonucleotide primer (TS), which serves as a substrate for telomerase; and 2) PCR amplification of telomerase activity product (an incremental 6 nt ssDNA ladder) with the oligonucleotide primer CX in a competitive amplification reaction with a 150 bp fragment encoding aa 97 - 132 of rat myogenin as an internal telomerase amplification standard (ITAS).

Details of the method are as follows: For the cells in culture, 10^5 cells were pelleted in culture medium. The supernatant was removed and the dry pellet stored at -80°C. The cells were lysed with 200 µl of ice cold lysis buffer consisting of 0.5% CHAPS, 10 mM Tris-HCl [pH 7.5], 1 mM MgCl2, 1 mM EGTA, 10% glycerol, 5 mM beta-mercaptoethanol, 0.1 mM AEBSF and left on ice for 30 min. The lysate was centrifuged at 14,000 RPM for 20 min at 4°C, and 160 µl of supernatant was collected into an Eppendorf tube making sure that no traces of the pellet were withdrawn; flash-frozen in an EtOH-dry ice bath and then stored at -80°C. Generally 2 µl of each lysate was analyzed containing the equivalent of approximately 1000 cells. In some instances the concentration of the protein in the extract was measured using the BCA protein assay kit (Pierce Chemical Company, Rockford, IL) and an aliquot of the extract containing approximately 6 µg of protein used for each telomerase assay.

Specificity of the processive 6 nt ladder is demonstrated by RNAse treatment. For RNAse controls, 5 µl of extract is incubated in 1 µg of RNAse (5'-3', Boulder, CO) for 20 min at 37°C. A 2 µl aliquot of extract is then assayed in 50 ul of reaction mixture containing 50 µM each dNTP, 344 nM of TS primer (5'-AATCCGTCGAGCAGAGTT-3'), and 0.5 µM of T4 gene 32 protein (USB, Cleveland, OH), [a^{32}P]dCTP, 5 attograms ITAS and 2 units of Taq polymerase (Gibco/BRL, Gaithersburg, MD) in a 0.5 ml tube which contained the CX primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') at the bottom sequestered by a wax barrier (Ampliwax™, Perkin-Elmer, Foster City, CA). After 30 min of incubation at room temperature for telomerase mediated extension of the TS primer, the reaction mixture is heated to 90°C for 90 seconds for inactivation of telomerase, and then subjected to 31 PCR cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds. The PCR products were electrophoresed on a 10% acrylamide gel as previously described (Kim *et al* 1994). Since human telomerase is processive, during the initial 30 minutes of incubation, in the presence of the TS primer, varying numbers of hexameric repeats are added to the primer and when subsequently amplified yield a 6-bp DNA incremental ladder. Extracts from the samples not containing telomerase do not extend the TS primer (Kim *et al* 1994). The 36 bp internal standard permits quantitation of relative telomerase activity levels by calculating the ratio of the internal standard to the telomerase ladder.

Telomerase Assay for Gross and Fine Needle Aspirates: Lysis of the gross tumor samples was performed as previously described (Piatyszek *et al* 1995: Kim *et al* 1994). Lysis of fine needle aspirates was performed as follows: FNAs were lysed as whole cell extracts in 50 µl of 0.5% CHAPS (Pierce Chemical Co., Rockford, IL) as per the normal lysis recipe but with the addition of 0.025U/µl of RNase inhibitor (5Prime 3Prime, Boulder, CO). New lysates were placed on ice up to 1 hour and the material was further broken by pipetting every 15 minutes. Extracts were then snap-frozen in liquid nitrogen and stored at -80°C until use. From the whole cell extracts of 60 µl, 5 µl (8%) were held back to determine estimate cell numbers within the individual FNAs. Detection of telomerase signal was determined by using the TRAP-eze™ Kit (Oncor, Gaithersburg, MD). Methods and sensitivity have been described by Holt et. al. (28).

Initial screens were performed as per the manufacturers instructions with the gross tumor samples. Due to the small amount of cellular material available from the FNAs, the TRAP assay was modified to utilize 5 - 10 µl of sample. This was done by decreasing the water content in reference to the respective sample volume increase.

Frozen Sections Telomerase Assay: A volume of 20 µl - 50 µl of a modified 0.5% CHAPS lysis buffer containing 1.5% glycerol (Sigma Chemical Co.) and RNase inhibitor (5Prime 3Prime) was added directly onto 5 µm thick frozen tissue sections fixed in OCT embedding compound (Miles, Inc., Elkhart, IN). This volume was then rigorously pipetted to resuspend and lyse tissues prior to running the telomerase assays. The lysate was then transferred to a 1.5 ml microfuge tube and snap frozen in liquid nitrogen. 2 µl were used per assay (Figure 1).

Phenol/Chloroform Extractions: In the presence of Taq polymerase or other nonspecific PCR inhibitors, as evidenced by a lack of the telomerase 6 bp ladder and the significant decrease or lack of the internal telomerase amplification standard (ITAS), a phenol and chloroform extraction method was performed. Briefly, sample extract was added to a cocktail consisting of TS primer, dNTPs, TRAP buffer and water, incubated at room temperature for 30 min to allow elongation of the telomerase products then heated to 100°C for 5 min to inactivate the telomerase. Tris-saturated phenol (USB, Cleveland, OH) vol:vol was added, the mixture centrifuged and the aqueous layer containing putative Taq or PCR inhibitors removed. This procedure was then repeated using a 24:1 chloroform: isoamyl alcohol solution (USB). Telomerase products were precipitated with 7M ammonium acetate and 2 volumes of 100% ethanol (USB) overnight. The samples were centrifuged at 14,000 rpm for 1 hr, rinsed with 70% ethanol, centrifuged a second time for 30 min - 1 hr and air-dried. At this point the TRAP reaction mixture was added with the corrected water volume and hotstart TRAP - PCR was performed without the 30 min incubation. Gels were run according to previously published methods (Piatyszek *et al*, 1995).

Strept-avidin Coated Magnetic Bead Retrieval Technique: An alternative method to using phenol/chloroform extractions on the inhibited samples, is a retrieval technique we have designed utilizing strept-avidin coated magnetic beads. This procedure is also useful for small

samples where the quantity is limiting and retrieval of the telomerase products must be efficient. The sample extract is incubated dNTPs and buffer. This solution containing telomerase elongation products is incubated for 30 min with a 1pmole solution of biotinylated C-rich oligomer. This complex is then mixed with the magnetic beads for 1 hr at room temp. After agitation, the products bound to the beads are melted off at 75°C for 15 min and immediately separated from the beads by a strong magnet. This solution is now added to a premixed cocktail containing the normal TRAP reagents appropriately concentrated to mix in the correct final concentrations. At this time , just the PCR reaction is performed. These products are run as described above on a 10% non-denaturing gel. Figure 2 illustrates a schematic of the protocol and a TRAP assay demonstrating the results.

Telomerase Extraction from Solid Tumor Samples: Partially thawed tissue samples were shaved on disposable petri dishes with sterile, disposable scalpel blades. The tissue shavings were immediately transferred to sterile 1.5 mL Kontes tubes (Vineland, NJ). 200 µL ice-cold lysis buffer with a final concentration of 0.5U/µL RNase inhibitor was added, followed by homogenization as previously described. Supernatant aliquots 200 µL were transferred to microfuge tubes, flash-frozen in liquid nitrogen, and stored at -80°C. To reextract samples, 200 µL of lysis buffer was added back to the sample pellet followed by another round of homogenization, centrifugation, and removal of supernatant.

Telomerase RNA and *In situ* Hybridization.

For telomerase RNA analysis, cells were prepared as follows: 50,000 cells were plated per chamber in a 2-chamber slide (Nalge-Nunc, Milwaukee, WI) and allowed to attach overnight. The slides were rinsed in PBS and placed in cold acetone for 20 min. Cells were then prepared for hybridization following established protocols . Briefly, cells were cross-linked in 4% paraformaldehyde (Sigma, St. Louis, MO), permeabilized in 0.05% Triton X-100 (Sigma), and deproteinized in 0.2N HCl (Sigma), deproteinized a second time with Proteinase K (1(g/ml) (Gibco), at 37(C. The cells were cross-linked again in 4% paraformaldehyde, followed by acetylation with 0.25% acetic anhydride (Sigma) in 0.1M triethanolamine (Sigma).

Probe Preparation. The template used to generate antisense and sense control probes consisted of the human telomerase RNA (hTR) (Geron Corp., Menlo Park, CA) cDNA sequence (560 nucleotides) in a pGEM-5Z plasmid . Single-stranded RNA probes labeled with [(^{35}S] UTP were synthesized according to manufacturer's instructions (Ambion, Austin, TX). Transcripts were alkaline hydrolyzed to generate an average length of 200 nt for efficient hybridization and purified with G-50 columns (Boerhinger Mannheim, Indianapolis, IN). After ethanol precipitation, the probes were resuspended in 30(l of 100mM DTT. Specific activity was approximately 3 x 10^7 cpm/(g of template DNA.

Hybridization and Washing. Hybridization solution consisted of 50% deionized formamide, 0.3M NaCl, 20mM Tris-HCl (pH7.5), 5mM EDTA, 10mM NaH_2PO_4 (pH 8.0), 10% dextran sulfate, 1x Denhardt's, 500µg/ml total yeast RNA, 10mM DTT and 50,000 cpm/(l of the ^{35}S-labelled RNA probe. Slides were hybridized overnight at 50(C. Washes were performed at 50(C in 5X SSC, 10mM DTT, then 65(C in 50% formamide, 2X SSC, 10 mM DTT. The cells were then washed twice in 0.4M NaCl , 10mM Tris-HCl (pH 7.5), 5mM EDTA before treatment with RNase A followed by washes in 2X SSC and 0.1X SSC. The slides were dehydrated in a graded ethanol series and dipped in Kodak NTB-2 nuclear track emulsion. After drying, the slides were stored in a light-tight box with desiccant at -80 C for 3-4 weeks. Slides were developed in Kodak Dektol developer, rinsed with water, fixed, rinsed again, counterstained with Gill's Hematoxylin (Fisher, Pittsburgh, PA) and coverslipped with Permount (Sigma). Photomicrography was performed on an Olympus BH-2 microscope using Kodak-400 *Elite* film.

Immunohistochemistry

Tissue Samples. Parrafin embedded 4 um histological sections representative of FA, carcinoma *in situ* (CIS) and invasive cancers were pulled and analyzed (MHS). These samples were chosen from results obtained from a prior study on telomerase activity in fine needle aspirates (FNA) and their gross sample counterparts. These blocks were serially sectioned and one set was H&E stained. The remaining sections were immunostained for the known breast cancer markers:

PCNA, PAI-1, CEA, p53, BCL-2, KI-67. *In situ* hybridization for the telomerase RNA component was performed on the remaining sections.

Immunohistochemistry results were based on a signal retrieval protocol. The sections were prepared as follows:

1. 4 - 5 um paraffin embedded sections were heated for 1 hr at 56°C, deparaffinized in 3 xylene washes and rehydrated in a graded ethanol series (100% - 30%).

2. Endogenous H2O2 was quenched by incubation un 3% H2O2 for 3 min at room temp. Then rinsed x2 in distilled water.

3. Slides were heated in 0.1M sodium citrate in a microwave pressure cooker for 8 min at 100% power (800 Watts). Then cooled at room temp for 30 - 60 min.

4. Slides were washed x3 in 1X PBS for 1-2 min. each

5. Antibodies were added to an antibody cocktail (1% BSA, 0.01% SDS, 0.1% Tween 20) as per the manufacturer's instructions and placed on the slide with enough volume added to cover the sample.

6. Place the slides in a moist chamber and incubate at 4°C for 18-24 hrs.

7. After incubation, allow slides come to room temp for 1 hr.

8. Remove slides individually and remove excess buffer. Rinse along the edge of the sample with 1 ml of 1x PBS, then wash in 1X PBS x3 for 5 min each.

9. For secondary antibody, the Vector kit catalog # 4002 was applied as per the manufacturer's instructions. And incubated in a moist chamber for 1 hr at room temp.

10. Wash x3 in 1X PBS for 3 min each.

11. Detection was obtained using Vector ABC elite (avidin-biotin complex) Reagent by placing slides in a moist chamber for 1 hr at room temp followed by 10 - 15 min incubation with the DAB staining reagent.

!2. Slides were rinsed in dH2O and counterstained with Gill's Hematoxylin followed by dehydration in a graded ethanol series (50% - 100%, 3 min each), cleared with xylene and coverslipped with Permount.

Antibodies: The antibodies used were as follows: MAb p53 clone DO-1, MAb PCNA clone PC10, MAb CEA, MAb PAI-1 (Oncogene); MAb Bcl-2 (Santa Cruz, cat # sc-509); PAb c-erbB-2 (cat# A 486) and PAb Ki-67 (cat # A 047) (DAKO).

Metaphase spread analysis

Methods for obtaining metaphase spreads were described previously (Aldaz *et al* 1989). Cultures were incubated with 0.01μg/ml Colcemid (Gibco/BRL, Gaithersburg, MD) in fresh medium for 4 h. After collection by trypsinization, cells were incubated for 1 h at 37°C in a 0.067M KCl hypotonic solution then fixed in 3:1 methanol:glacial acetic acid, rinsed and spun 2X for 5 min each at 1200 RPM . After resuspension in 1-2ml of fix, pellets were dropped onto precleaned microscope slides and stained with Giemsa Stain (Sigma Chemical Company, St. Louis, MO). Chromosomes were counted from >25 randomly chosen metaphase spreads.

Fluctuation analysis

The frequency of escape from crisis (e.g. immortalization frequency) was estimated using an approach based on what is essentially a fluctuation analysis previously described (Shay *et al* 1993). Clones were expanded several population doublings before crisis into multiple series in several sizes of culture dishes at a constant cell density. Each series was subsequently maintained as a separate culture, so that at the end of the experiment the fraction of each series that gave rise to an immortal cell line could be determined. Using different size dishes permitted series to be set up which contained a different number of cells per dish while maintaining a constant culture environment (cells/cm^2). Cultures were split at or just prior to confluence. Once cells reached crisis, they were split (at least once every 3 weeks) until only a few surviving cells remained or the culture had immortalized. Immortalization was expressed as the number of immortal lines per number of culture series. Frequency is expressed as the probability of obtaining an immortal cell line based on the total number of independent immortalization events and dividing by the total number of cells plated.

Results

This section contains the results obtained over the last working year of the grant.

A. Results directly pertaining to the Statement of Work.

Mixing experiments were conducted with normal HME 31 and 32 cells and HME 31 and 32 cells infected with p53 mutant 143. The ratio of mixing was 10:1 normal cells to healthy cells with the hypothesis that the mutant p53 may confer a selective growth advantage over the normal cells. To this end, 90,000 normal cells were mixed with 10,000 infected cells in a 15 mL tube with medium and plated onto a 100 mm tissue culture dish. At the time of passage, the cells well harvested with trypsin and counted. 100,000 of the resulting cells were plated onto glass chamber slides (Nalge-Nunc) and allowed to settle overnight. Of the remaining cells, 200,000 were plated onto 100 mm dish and continued *in vitro* passaging. Slides were fixed in 70% methanol. The cells in the chamber slides were immunostained with an antibody recognizing the mutant protein conformation using the horseradish-peroxidase staining kit from Vector Labs and immunohistochemistry was performed as per the manufacturer's instructions. Figure 3a is a graph representing the growth curves over 25 PDL for HME 31 and 20 PDL for HME 32. The results from the growth counts and the immunohistochemistry counts indicate that there is no selective growth advantage for the cells containing the mutants while the cells are young and fast growing. As they approach senescence the difference becomes more significant. At approximately 10 PDLs from crisis, the number of cells exhibiting mutant p53 increases in proportion to the overall population. By the time the cells are in crisis, the remaining proliferating cells are those containing the mutant p53 protein. No cells immortalized, but extension of *in vitro* lifespan was observed for both the cell strains tested. Figure 3b illustrates the increase of mutant containing cells. One drawback to this type of analysis is that other p53 mutations could be occurring as the cells senesce and become more karyotypically unstable. However, the take home message is that no growth advantage is observed for the mutant cells until the population approaches senescence.

In reference to whether or not mutant p53 expression is necessary to maintain cell growth, a 2-part experiment was conducted. The first part of the experiment involved the immortal clone HME 32

(273) which was obtained during the first grant year. Wild type p53 was reintroduced to this clone in the form of a retroviral expression vector. Figure 4a graphically shows the PDL over 25 passages and 4b is a representative TRAP gel demonstrating telomerase activity concurrent with the 25 PDL in culture. As observed, after an initial decrease in the growth rate, there was no significant effect on either growth rate or telomerase activity. This may be due to the dominant-negative effect of the p53 mutant 273 in the cells. Normal p53 function appears to be abrogated. In order to observe whether p53-associated proteins might cause a cell-cycle arrest, in the case of normal, but non-functional p53 protein present, the immortal clone was infected with a defective retrovirus containing p21 (Cip 1). As demonstrated by the growth curves in figure 5a and the photomicrograph in figure 5b, there was no effect on growth. Interestingly, when the cells were allowed to become overconfluent, the levels of p21 increased and the cells remained sensitive to contact inhibition.

In keeping with the question of whether mutant p53 expression is necessary for the continued proliferation of the cell, a tetracycaline-inducible p53 promoter was cloned into spontaneously immortalized, telomerase positive LFS fibroblasts. Introduction of wild type p53 utilizing an inducible tet-promoter was performed in order to explore the possibility of a direct correlation between p53 and telomerase activity. Immortal 041 fibroblasts were transfected with a wild type p53 cDNA driven by a tetracycline repressible promoter (Gossen & Bujard, 1992). As seen in Fig. 6a, when tetracycline is removed from the media, p53 is strongly induced and cell growth is arrested. Telomerase activity results show a >10 fold decrease in telomerase activity over a 7 day period (Fig. 6b). These data are consistent with previous observations (Holt *et al* 1996) ;(Holt *et al* 1997) demonstrating that in telomerase competent cells, exiting the cell cycle results in downregulation of telomerase activity.

A concurrent experiment was conducted using somatic cell hybrid technology. Somatic cell hybrids were made between 041 (tel+, p53-) X HT1080 (tel+, p53+) as well as 087 (tel-, p53-) X HT1080(tel+, p53+). We wanted to determine if the cell hybrids would continue dividing, undergo cellular senescence, or growth arrest. Somatic cell hybrids were formed between a

telomerase positive, hygromycin resistant fibrosarcoma cell line HT1080, expressing wild type p53, and tumorigenic *ras*-transformed 041 (tel +) and 087 (tel -) fibroblasts containing the gene for neomycin (G418) resistance. Hybrid clones resistant to both hygromycin and G418 readily formed from crosses between HT1080 (tel+, p53+) cells and tumorigenic *ras*-transformed 087 (tel-, p53-) cells but very poorly between HT1080 (tel+, p53+) and tumorigenic *ras*-transformed 041 (tel+, p53-) cells (Table 1). The three HT1080/041*ras* cell hybrid clones that formed were telomerase positive but senesced rapidly. All of the hybrid clones tested (10/10) between 087*ras* (tel -) and HT1080 (tel +) were telomerase positive and grew indefinitely.

In order to determine whether the loss of wild type p53 is sufficient for the reactivation of telomerase activity, DNA prepared from LFS fibroblasts was analyzed at various passages for p53 mutations by single strand conformational polymorphism (SSCP) and protein extracts for telomerase activity. In all LFS fibroblasts examined, the remaining wild type p53 allele was lost (041 cells) or replaced with a mutant p53 allele (087 cells) by PDL 67 and absent from all subsequent PDLs (data not shown). Cell protein extracts from these fibroblasts were analyzed for the presence of telomerase activity at various PDLs during their growth in culture and for loss of p53 protein by Western blot. We observed that at PDL 19, 26, 30 and 35, 041 cells had no telomerase activity and p53 was still present. At PDL 54, 041 fibroblasts had lost p53 protein and gained detectable telomerase activity that was continuously maintained (Fig. 7). Since 041 fibroblasts prior to PDL 54 did not have detectable telomerase and lost p53 protein at approximately the same time PDL as telomerase reactivation (Fig. 7), the direct loss of the remaining functional wild type p53 allele appears to correlate with reactivation of telomerase activity.

In 6 of 6 independent immortalization experiments, the 087 cell lines never developed detectable telomerase activity. It has been reported (Tsutsui *et al* 1995) that enhancement of the immortalization frequency of 087 could be obtained by treatment of cells with aflatoxin B1 (AFB1). Thus, an additional immortalization study with cells derived from 087 + AFB1 was undertaken and cells capable of indefinite proliferation were also found to be telomerase negative

(Bischoff *et al* 1990) (Fig 8A). At the time of immortalization, DNA extracts from these cells were examined by SSCP and were found to be homozygous for the p53 mutation at codon 248 (data not shown). Mixing cellular extracts from telomerase negative (087) cells with an extract from a telomerase positive cell line (HT1080) did not result in inhibition of telomerase activity (data not shown) indicating that 087 cells did not contain a diffusable telomerase inhibitor. These data (summarized in Table 2) indicate that the reactivation of telomerase activity during the immortalization of LFS skin fibroblasts may be cell strain specific.

TRF Analysis. The telomere lengths in the LFS skin fibroblasts were estimated using TRF (terminal restriction fragment) analysis (Shay *et al* 1994) to determine if reactivation of telomerase activity in 041 cells resulted in telomere length stabilization, as well as to determine the telomere dynamics of the telomerase negative 087 cells (Fig. 9). In the 041 cells, a progressive loss of telomeric DNA from 8 kb was observed up to the time of detection of telomerase activity (PDL 54), followed by stabilization of telomere length at 2.5 kb. In the telomerase negative 087 cells, TRF analysis showed two prominent populations of telomere lengths at approximately 10 kb and 3 kb. As the cells progressed in culture, the 3 kb band remained stable while the 10 kb band decreased in length until there was no discreet signal observed. With continued culture, a subset of telomeres in these cells were again observed with an increase in length to 10 kb (Fig. 9). These results are consistent with the telomere length fluctuations as reported by Murnane *et al* (1994).

As a part of the US Army funded Breast Tissue Repository, we were able to establish a number of normal mammary epithelial and stromal cell strains in conjunction with tumor-derived cell lines from the laboratory of Dr. Adi Gazdar. During the establishment of these lines, telomerase activity was assayed at different time points in culture. We observed that the cells appear to be quiescent for months in culture and then, presumably, after the derepression of telomerase, cell proliferation recommences. Table 3 presents telomerase activity along with p53 status for the cell lines in which normal epithelial and stromal cell strains and tumor-derived lines from the same patient were established. TRAP analysis was performed on the cell lines and the results are presented in Figure 10a. As shown in Figure 10b, telomere lengths of the normal cell strains are longer than those of

the corresponding telomerase positive immortal cell lines. In half the cell lines established with corresponding normal cell strains, by inmunohistochemistry p53 was abrogated either by overexpression or not present (data not shown). Figure 10c illustrates the RNA component of telomerase as detected by in situ hybridization using a radio-labeled probe. Interestingly, the telomerase negative 087 cell lines did not have any signal detectable above background for the RNA component. In comparison, the telomerase positive 041 cells had stong telomerase RNA signal after immortalization (re-activation of telomerase) In a manuscript being prepared by Dr. Gazdar's group, the cellular and molecular markers analyzed in the cell lines parallels the results obtained from the gross tumor specimen. Thus, detailed results of these findings will be forthcoming. This is an important finding for *in vitro* cell culture models of breast carcinogenesis.

B. The following is a compilation of results stemming from related projects in the field which are indirectly related to the Statement of Work presented in this grant.

To further confirm the existence of a relationship between the inactivation of p53 and the reactivation of telomerase activity, a series of experiments was conducted which involved the LFS mammary epithelial cell strain HME 50. This cell strain has a mutation at codon m133t in the p53 protein. Thus it was a good strain to use because only one extra hit was necessary to inactivate the wild type p53 protein. Consequently, culture time was reduced. From this cell strain, clones were isolated and telomerase activity as well as telomere length was analyzed. The clones were passaged concurrently to determine if crisis occurred at the same time or if clonal variation in proliferative capacity existed. Figure 11a &b illustrates the TRF analysis results along with the TRAP results. Into these clones HPV 16 E6 was introduced via transduction at approximately 15 PDLs before crisis. Telomerase was assayed at the next round of subculturing. The results are illustrated in figure 11c. Telomerase was reactivated within the next population doubling. Thus, presumably by degrading the remaining wild type p53 allele, somehow repression of telomerase was deregulated and the cells progressed to the immortal phenotype.

A series of primary human breast tumor specimens was obtained to determine if the NP-40/NaDOC lysis buffer could also be utilized to enhance extraction of telomerase activity from tumor tissues. Tumor samples were homogenized and processed in either the CHAPS or the NP-40/NaDOC lysis buffers (Piatyszek *et al*, 1995) (Figure 12, Table 4). In the samples examined, no clear difference in extraction of telomerase activity between the detergents was observed using NP-40/NaDOC, contrary to the results seen in cultured cells. This is possibly due to the homogenization step that is included in the preparation of solid tumor samples. Using either CHAPS of NP-40/NaDOC extraction, only 39% (7/18) of the tumor tissue samples examined were positive for telomerase activity in the initial extractions, possibly due to the presence of PCR inhibitors (Figure 12, ILC, first extraction). We found that 17/18 of these were positive for telomerase activity in the second extraction and all 18 were positive by the third extraction (Table 4).

In a recent study, we found that approximately 50% of fibroadenomas were positive for telomerase activity. In order to determine whether there existed a correlation with other known breast cancer markers, immunohistochemistry was applied to paraffin embedded sections. Serial sections were stained with known breast cancer markers: proliferating cell nuclear antigen (PCNA), c-erbB2 (c-neu), bcl-2, ki-67 (mib-1), Plasminogen antigen inhibitor (PAI-1), carcinoembryonic antigen (CEA) and p53. There appears to be a correlation with the telomerase positive fibroadenomas and Ki-67 staining. Ki-67 is a proliferating cell marker for all phases of the cell except G2. The occurrence of the marker and telomerase activity may coincide with the presence of cyclin B1. Figure 13 is a composite of the telomerase positive fibroadenomas and Ki-67 staining.

Along the same idea, in collaboration with Dr. Gazdar's group, we looked at breast carcimonas *in situ* . These lesions are generally considered preneoplastic due to the fact that they are still localized within the basement membrane and have not yet infiltrated the surrounding tissue. Telomerase activity was assayed and the results showed that individual CIS were variable for the intensity of telomerase activity (Figure 14).

In conjunction with this study and the other aforementioned studies, it was of particular interest to investigate the status of the RNA component of telomerase in these sections as well as the other cells studied. To that end, *in situ* hybridizations were performed on cells in culture as well as the paraffin sections. A plasmid containing the RNA component of telomerase was radio-labelled and hybridized to the cells or the sections. After stringent washes, the slides were dipped in Kodak nuclear emulsion and exposed for 3 weeks. After development, the slides were photographed. The slide results showed that normal cells had no detectable telomerase activity and immortal cells that were telomerase positive for the protein also had detectable RNA signal. Figure 15 demonstrates the telomerase RNA signal observed from a representative normal breast tissue and a fibroadenoma. Interestingly, the CIS samples demonstrated variable telomerase RNA signal intensity between adjacent lesions (Yashima *et al*, 1997)). One important observation was that the signal intensity of CIS adjacent to overt carcinomas was generally strong and micrometastises appeared within the surrounding tissue negative for signal. The fibroadenomas were all weakly positive regardless of the telomerase activity. It is important to note that FA positive for telomerase activity were generally 10-fold less intense than signal from overt breast cancers. Another important observation was the fact that normal proliferating breast tissue has weak telomerase RNA signal detectable in the epithelial portion of the tissue. This may reflect stem cell origins in the epithelial compartment such as the myoepithelial cells which may differentiate to either basal or luminal epithelial cells. Whether this is restricted to the breast system or reflects other similar epithelial-stromal tissues needs further investigation.

In view of the results with the fibroadenomas, which is a hyperproliferation of the stromal component in the breast, we wondered what type of effect co-culture of HME and HMS cells from the same patient would produce. After a series of preliminary experiments, a medium was found which would sustain both the HME and the HMS cells. It consisted of fibroblast basal growth medium supplemented with the HME growth factors and did not contain serum. HME 32 and HMS 32 were co-cultured in 6 well plates with the stromal cells grown on the insert layer for 2 reasons: 1). Theoretically any supernatants supplied by the stromal cells would reach the epithelial

cells more effectively by gravity and 2). The stromal cells are much easier to detach from the plate or insert, so it was much easier to get accurate cell counts. From the data (Figure 16) it appears as though the stromal cells act as proliferation regulators of the epithelial cells.

Other differences in the cell lineages of this system are evident at hyperoxic and hypoxic conditions. HME 31 and HME 50 cells; HMS 50 and IMR 90 (normal diploid lung fibroblasts) cells; HME 50 E6/E7, HMS 50 E6/E7 immortalized cells; and HME 50 spontaneously immortalized cells were maintained in either 1% oxygen, 20% (normal) oxygen or 40% oxygen conditions. Figure 17 graphically depict the growth curves. Normal fibroblasts stop proliferating (senesce) within 14 days. Mortal epithelial cells continue until normal senescence occurs. Interestingly, after cells have immortalized the cell type does not appear to be a factor as the cells continue to grow without plateauing.

Discussion

We found that clones from LFS fibroblast cell strains can spontaneously immortalize utilizing either a telomerase dependent or independent mechanism. While telomerase positive immortal LFS cells have stable telomere lengths and high levels of the telomerase RNA (hTR) component, immortal telomerase negative LFS cell lines have long and heterogeneous telomere lengths and no increase in hTR levels above corresponding preimmortal cell strains. This suggests that at least in LFS, some cell strains are committed at an early point to a telomerase positive or telomerase negative immortalization pathway.

Our results using LFS fibroblast cells also demonstrates that activation of telomerase activity is not a requirement for immortalization or tumorigenicity since *ras*-transformed 087 cells formed tumors in nude mice without reactivation of telomerase activity. The 087 LFS skin fibroblasts do not appear to require a functional telomerase since they maintain their telomeres by an alternative lengthening mechanism.

The TRF analysis in 087 cells revealed 2 major populations of telomeres. Since the lower band remains unvarying in length throughout culturing, this may be indicative of a stable telomere

population with the longer telomere population undergoing recombinational events and breakage-fusions-bridge cycles. Alternatively, the shorter telomere population in these cells may serve as the minimum number of TTAGGG repeats required for telomeric function. As the telomere size decreases below this threshold, a signal may be sent to begin lengthening the telomeres by an as yet unknown mechanism (Lansdorp *et al* 1996). Another possibility for the observed heterogeneous telomere length and lack of telomerase activity could involve telomeric binding proteins as described by Chong (Chong *et al* 1995). For example, telomeric binding proteins may prevent the longer telomeric population from undergoing recombinational events that would maintain that length. As the telomeres shorten, the lack of bound proteins could either allow access of the ends to recombinational events or signal a negative feedback loop that initiates recombination to increase the telomere length.

Expression of exogenously transfected wild type p53 into 041 (tel +) cells resulted in growth arrest with a corresponding inhibition in telomerase activity. However, these results do not indicate that there is a direct interaction between wild type p53 and telomerase activity, merely that telomerase activity is downregulated in cells exiting the cell cycle (Holt *et al*, 1997). This is consistent with previous observations that show that quiescent cells have reduced telomerase activity (Buchkovich & Greider, 1996; Holt *et al* 1996).

Tumorigenicity studies in nude mice of *ras* transformed 041 and 087 cells showed that even with tumor formation, the status of telomerase activity did not change. Whether there is a difference in metastatic capability and telomere status requires further investigation. Immortal 041 cells that have a p53 mutation and express only a 184aa portion of the protein may have the remaining components of the p53 pathway intact, thus leading to complementation and growth suppression in the wild type expressing HT1080 x 041 hybrids. (Bryan *et al* 1995)reported that some somatic cell hybrids between telomerase positive and telomerase negative cell lines recommenced proliferation after exhibiting cellular senescence. Unlike (Bryan *et al* 1995) we did not observe reversible cellular senescence in the somatic cell hybrids generated in this study. In contrast, the continued proliferation capability of the HT1080/087 hybrid clones may be due to the

presence of the p53 mutant 248 acting as a dominant negative, abrogating the wild type p53 function of the HT1080 cells. However, the poor efficiency of generating HT1080/041 hybrids may indicate differing mechanisms of complementation for inactivation of the p53 pathway. Because 041 cells are essentially null for p53, the introduction of the wild type p53 from HT1080 cells may cause a cell cycle arrest that remains separate from telomerase activity.

The results from the somatic cell hybrid experiments taken together with the TRF results is consistent with the hypothesis that the wild type p53 expressed in the HT1080/087 hybrid clones may recognize the long and heterogeneous population of telomeres as damaged DNA resulting in a checkpoint growth arrest. The resulting effect would be an active telomerase which cannot bind to and replace telomeric TTAGGG repeats and thus telomeres continue to shorten. As the telomeres reach a critical length, p53 may have limited telomere accessibility due to conformational changes, or there may be an unknown mechanism that would then allow telomerase to outcompete p53 for the telomeric ends by somehow superseding the p53 binding strategy.

The ALT pathway does not appear to be frequent in primary tumors. It can be observed when viral oncoproteins (e.g. SV40 large T antigen) may predispose certain cells (primarily fibroblasts) to the ALT pathway. Thus, in rare instances, the viral oncogene would integrate into a region(s) that would disrupt the activation of telomerase, such as the RNA component of telomerase or the functional telomerase gene. Our data suggests that such a hypothesis is unlikely since the immortalized cell lines obtained in the present study were spontaneous events. Possibly, it is cell strain specific genetic mutations since we have shown in this study and previous studies (Shay *et al* 1995) from patients with p53 germline mutations, the immortalization pathways are consistently and reproducibly different in their activation of telomerase activity, telomere structure, and telomerase RNA expression.

Conclusions

Key accomplishments:

1. First report: spontaneously immortalized Li-Fraumeni HME cell strain

2. First report: human mammary stromal strain infected with HPV 16 E7 immortalized.

3. First report: human mammary epithelial strain immortalized with a p53 mutant.

4. First report on the relative effectiveness of different promoters for achieving extension of lifespan and / or immortalization.

5. Report on the effectiveness of different p53 mutants in extension of lifespan and immortalization.

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TABLE 1. Summary of mutation analysis of endogenous p53 in fibroblast skin cells obtained from patients with LFS

Cell Strain	p53 mutation	Population Doubling	Telomerase Activity
041	184FS	<36	-
041	184FS	>54	+
087[a]	R248W	19-166	-
087ras[b] Tum^-	R248W	59	-
087ras[b] Tum^+	R248W	104	-

[a] six independent immortalization events
[b] two independent transfectants

TABLE 2. Summary of somatic cell hybrid colony formation and telomerase activity between HT1080 cells and 087 or 041 cells

Cell Line	Efficiency[a]	# Colonies Tested by TRAP	Telomerase Activity
HT 1080	NA	NA	+
087ras	NA	NA	+
041ras	NA	NA	-
HT1080 x 087*ras*	250	10/10	+
HT1080 x 041*ras*	3	3/3	+

[a]number of hybrid colonies formed

Table 3. Telomerase activity and p53 status of the breast tumor-derived cell lines from The Lab of Dr. Adi Gazdar. These are lines with which corresponding normal cell strains have been established.

Tumor Cell Line	Telomerase Activity	p53 Status*
1419	+	++
1500	+	+
1569	+	-
1739	+	+

* p53 status was determined by immunohistochemisrty.

Table 4: Summary of the breast tumors re-extracted in order to Remove PCR inhibitors from the TRAP reaction.

Tumor #	1st Extraction	2nd Extraction	3rd Extraction	Pathology
1	-	+	+	LCIS
2	+	+	+	SCC
3	+	+	+	IDC
4	+	+	+	ILC
5	-	+	+	ILC
6	-	+	+	ILC
7	+	+	+	IDC
8	-	-	+	IDC
9	-	+	+	IDC
10	-	+	+	IDC
11	-	+	+	IDC
12	-	+	+	IDC
13	+	+	+	IDC
14	+	+	+	IDC
15	-	+	+	IDC
16	-	+	+	IDC
17	-	+	+	IDC
18	+	+	+	IDC

LCIS - lobular carcinoma in situ; SCC squamous cell carcinoma; IDC - invasive ductal carcinoma; ILC - infiltrating lobular carcinoma.
No. Specimens Telomerase positive in 1st extraction 7/18 (39 %)
No. Specimens Telomerase positive in 2nd extraction 17/18 (94 %)
No. Specimens Telomerase positive in 3rd extraction 18/18 (100%)

Figure Legends

Figure 1. Schematic representation of TRAP assay lysis process for frozen histological sections. Twenty to 50 µl of 0.5% CHAPS lysis buffer containing 1.5% glycerol is placed on the cryosection and vigorously resuspended. The sample extract is then placed in a 1.5 ml microfuge tube and snap frozen. 2 µl is used per assay as previously described (Piatyszek *et al* 1995).

Figure 2. Schematic representation of the protocol used to enhance for the products of telomerase extension reaction in the presence of inhibitors. The sample extract is mixed with a cocktail containing dinucleotides and the forwrd primer which also serves as the telomerase substrate. After the standard 30 minutes, this cocktail is mixed with a biotinylated c-rich oligomer for 30 minutes at room temperature. After binding of the tagged oligomer with the reaction products, this cocktail is then hybridzed to magnetic beads coated with strept-avidin for 1 hour. The inhibitors are then removed by aspirating the supernatant and resuspending in fresh buffer. The products of the telomerase reaction are then melted off the beads in a 75°C water bath. The products are immediately placed in a cocktail of components that are PCR ready. At this point just the amplification step occurs. The products are run on a PAGE gel as per the standard protocol. b. Representative gel before and after retrieval using strept-avidin coated magnetic beads. ITAS = internal telomerase assay standard. Before and after - before and after bead retrieval.

Figure 3. a. Growth curves for HME 31 and 32 control cell strains and strains infected with p53 mutant 143. As observed from the graph, there is no selesctive growth advantage early in the in vitro lifespan of the population. As the population reaches cellular senescence, the cells containing the mutant p53 show an advantage in number of divisions. Figure 3b illustrates the increase in the number of cells with mutant p53 in the population as the cells near crisis.

Figure 4. a. Growth of the immortal clone HME 32 (273) is presented over 25 PDL in culture after introduction of a wild-type p53 by defective retroviral infection. After an initial decrease which may also been attributed to the cells recovery from the infection process, there was no change in the growth rate of the cells. b. The TRAP gel demonstrates the telomerase activity over the concurrent time in culture. There is no apparent drop in telomerase activity. Quantitation revealed an initial difference of approximately 2-fold. This difference was quickly decreased as the cells recovered from the transduction process. Thus, at this time, whether this event was due to the wild-type introduction, the infection protocol or the mutant wild-type acting as a dominant - negative needs further investigation.

Figure 5. Growth curves for immortal clone HME 32(273) as a control strain and with the introduction of p21 (Cip1) by defective retrovirus. p21 showed no effect in the growth of the immortal clone. Perhaps due to the fact that the mutant p[53 was not causing a cell cycle arrest and therefore not signalling for the activation of p21. Thus an excess of p21 does not appear to have an inhibitory effect on the cells immortalized with p53 mutant 273.

Figure 6. Growth analysis and telomerase activity of 041 cells transfected with wild type p53 and a tetracycline-inducible promoter system. In the presence of tetracycline, wild type p53 is expressed. (a) growth curve in terms of numbers (10^6) of cells over 14 days in culture. Test groups include: [- p53, + tet]; [- p53, + tet, 0.75% serum]; [+p53, - tet]. (B) TRAP gel results of the same test sets as in (a). b. TRAP activity assayed in the presence of induced wild-type p53 over a seven day period. Quantitation revealed a >10 fold decrease in telomerase activity as a function of the internal standard over time in culture.

Figure 7. Telomerase activity and p53 protein expression levels in 041 and 087 cells. (A) TRAP gel showing results of telomerase activity in 041 and 087 cells precrisis (PDL 19) and postcrisis (PDLs 54 and 64, respectively). Aflatoxin B1 (AFB1) clones were obtained from AFB1 induced immortalization of 087 cells. Clones shown here were obtained after 100 PDLs in culture.

Negative control is lysis buffer and positive control is HT1080 cell extract. (B) p53 immunoblot showing the levels of p53 in 041 cells (p53 null) precrisis (PDL 46 and 43 respectively) and postcrisis (>PDL 56). Note that there appears to be lower levels of p53 in 041 cells at PDL 56 perhaps indicating selection for the telomerase positive cells which in this case, are also p53 null by SSCP. Control cells: IMR90, normal diploid lung fibroblasts; 1299, non-small cell lung carcinoma derived, telomerase postive, p53 null immortal cell line.

Figure 8. Comparative telomerase activity of *ras*-transformed, tumorigenic 087 and 041 cells from nude mice. After resection of the tumors, cultures were re-established and cells harvested for telomerase activity at increasing PDLs. Negative control is lane is lysis buffer. 087 cells continue to remain telomerase negative regardless of tumorigenicity while 041 cells remain telomerase positive.

Figure 9. TRF analysis of 041 cells and 087 cells during increasing PDLs. The pattern of telomeric signal is similar in the spontaneously immortalized cell lines (*-ras*) as well as the *ras*-induced immortalized cell lines (*+ras*). While the initial precrisis 041 cells exhibit 2 major telomere subpopulations (9kb and 3.5 kb), the larger is lost and it is a shorter subpopulation that determines the stabilized telomeric lengths. The 087 cells retain 2 primary subpopulations of distinct telomeric lengths (10kb and 3kb). At PDL 95 however, the larger subpopulation disappears and again re-establishes a 10 kb signal by PDL 155.

Figure 10. TRAP activity analysis and TRF analysis of normal HME and / or HMS cell strains with their respective tumor derived cell line. a. Telomerase activity was assayed at different time points in culture as indicated by the PDL of each sample lane. b. TRF analysis of these same normal HME and HMS cell strains with the corresponding tumor derived lines.

Figure 11. TRF and TRAP analysis of clones isolated from cells obtained from a LFS patient (HME 50). This cell strain exhibits a mutation in the germ-line for the p53 gene at codon 133 (M →T). b. TRAP assay of these same clones at the next harvested PDL. The telomerase activity has been re-activated after one PDL. Thus, somehow the degradation of the remaining wild-type p53 allowed the re-activation of telomerase by an as yet undetermined interaction.

Figure 12. Re-extraction of tumor samples allows for the liberation of telomerase without the presence of contaminating inhibitors to the PCR step. Different breast tumors were re-extracted for telomerase activity and assayed after each extraction. In those samples that contained inhibitors, by the third extraction, the inhibitors were eliminated. In those tumors in which telomerase activity was detected from the initial extraction, telomerase activity was still observed after the third extraction indicating the amount of free telomerase in the cells is greater than suspected and that the lysing techniques are not efficient in extracting all the available telomerase.

Figure 13. a. Representative TRAP gel demonstrating the telomerase activity in breast fibroadenomas. RNase incubated samples were included as controls to show RNase sensitivity of the telomerase products. Photomicrograph of Ki-67 positive - telomerase positive FAs. 1. Ki-67 positive immunostained section (telomerase positive); 2. Ki-67 negative immunostained section (telomerase negative).

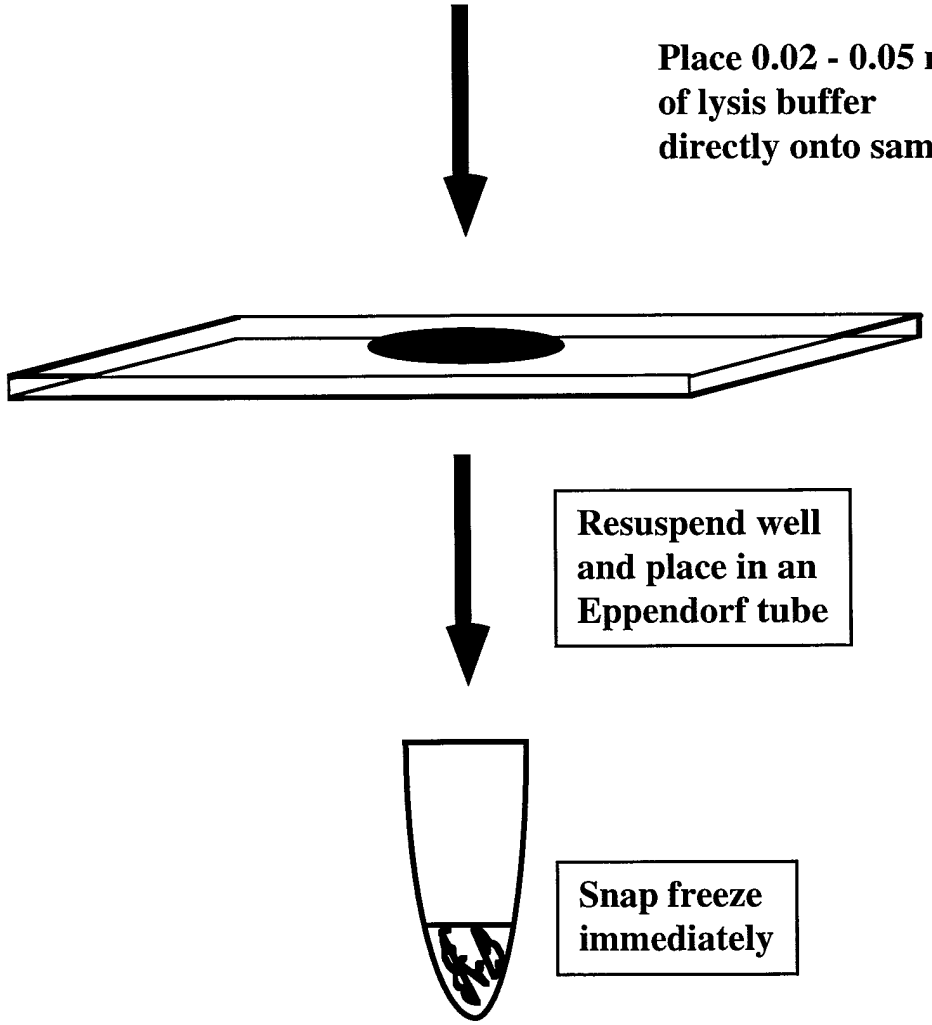
Figure 14. Photomicrograph of a histological section from normal breast tissue and from a fibroadenoma. a. normal breast tissue under bright-field conditions. b. the same section using dark-field conditions. Note the intensity of signal in the area of the ducts (epithelial component) with very little signal observed in the stromal component. c. representative fibroadenoma under bright-field and (d) dark-field conditions. It is interesting to observe that although fibroadenoma is defined as a hyperplasia of the stromal cells, there is no corresponding upregulation on the stromal compartment and the relative intensity of signal is still concentrated in the epithelial cells (ducts).

Figure 15. Composite of representative telomerase RNA (hTR) signal in 041 cells and 087 cells precrisis and postcrisis. 041 cells and 087 cells precrisis showed no telomerase RNA signal above background levels (A and C). (B) are 041 cells postcrisis and (D) are 087 cells postcrisis. Note the intense hybridization signal (arrow) for 041 immortal cells (B) in contrast to the 087 RNA signal comparable to that of the preimmortal cells (D).

Figure 16. Graphic representation of the co-culture of mammary epithelial and stromal cells. HME 50 and HMS 50 were co-cultured in 6-well plates for a period of 3 weeks. Different confluencies of cells were used the optimal co-culture conditions. Stromal cells appear to act as proliferating agents in the overall growth of the epithelial cells. In all cases the population doublings of the epithelial cells remained constant. However, the stromal cell growth varied with plating density. The possible effects of the epithelial cells on the stromal cells may reflect what happens *in vivo* in the case of fibroadenoma where there is a hyoerorolifewration of the stromal compartment in the breast tissue.

Figure 17. Growth curves for mammary epithelial and stromal cells under different oxygen conditions. As can be observed from figures a and b, the stromal cells die after 14 days in 40% oxygen. In contrast, the epithelial cells (c and d) continue to divide as in the control group. Interestingly, when the cells are immortal, no distinguishing difference can be made between the cell lineages (e and f).

Detection of Telomerase Activity from Frozen Sections



Use 2μ l of sample per TRAP assay

Figure 1

Telomerase Detection in the Presence of Taq Polymerase Inhibitors Using Streptavidin Coated Magnetic Beads

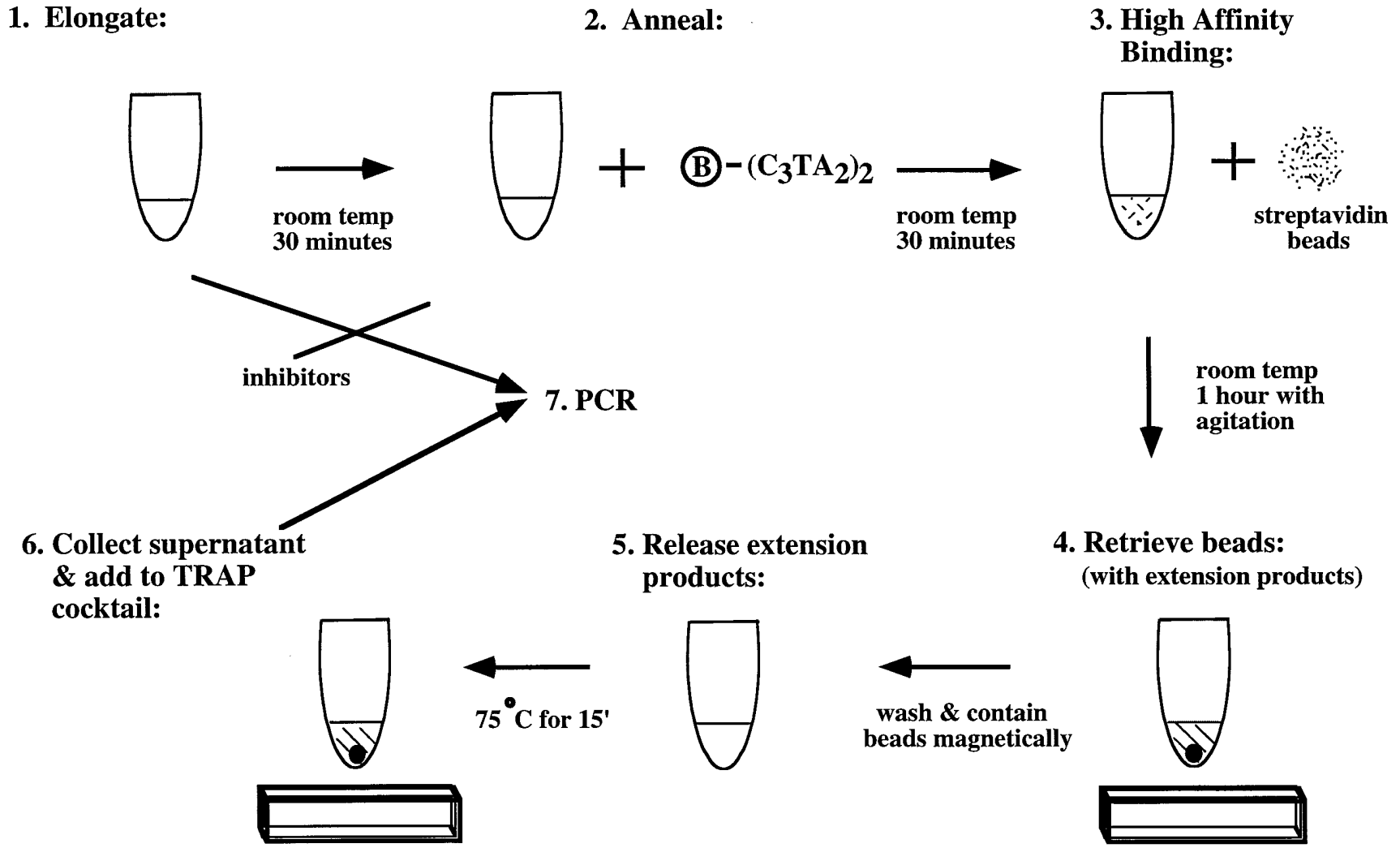
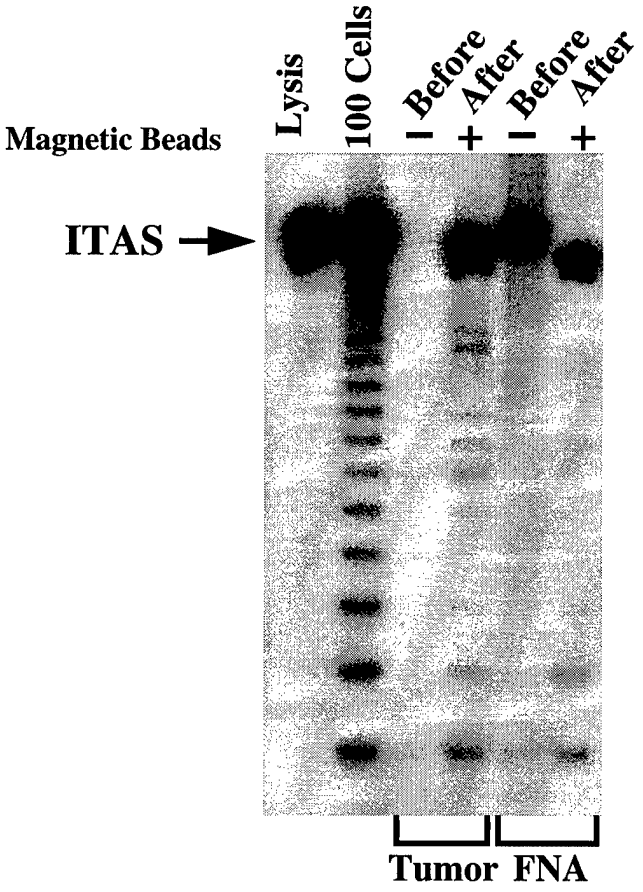


Figure 2

Figure 2b


Magnetic Beads
Lysis
100 Cells
Before
After
Before
After
-
+
-
+
ITAS
Tumor
FNA

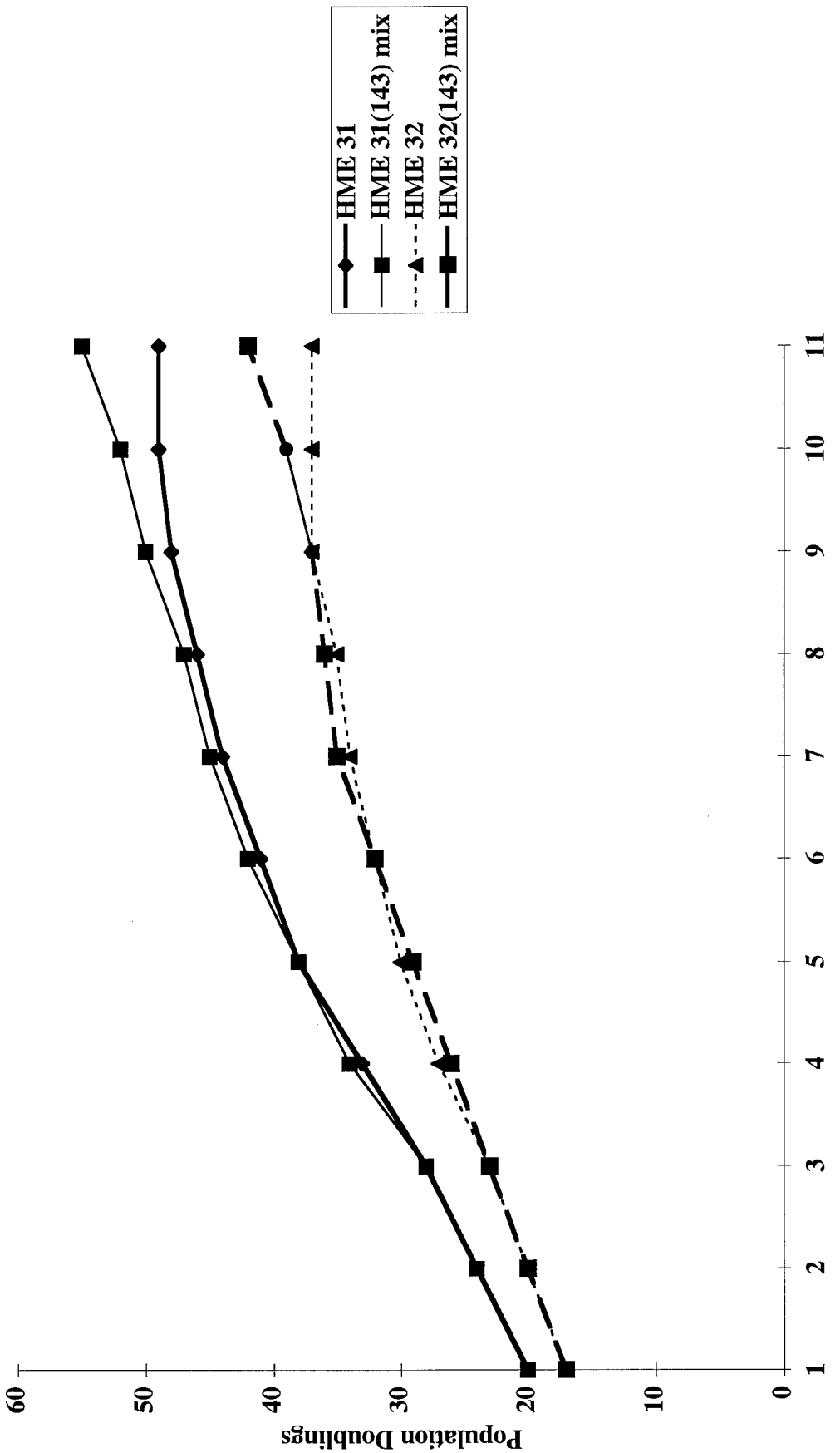

HME 31
HME 31(143) mix
HME 32
HME 32(143) mix
Population Doublings
0
10
20
30
40
50
60
Weeks in Culture
1
2
3
4
5
6
7
8
9
10
11

Figure 3a

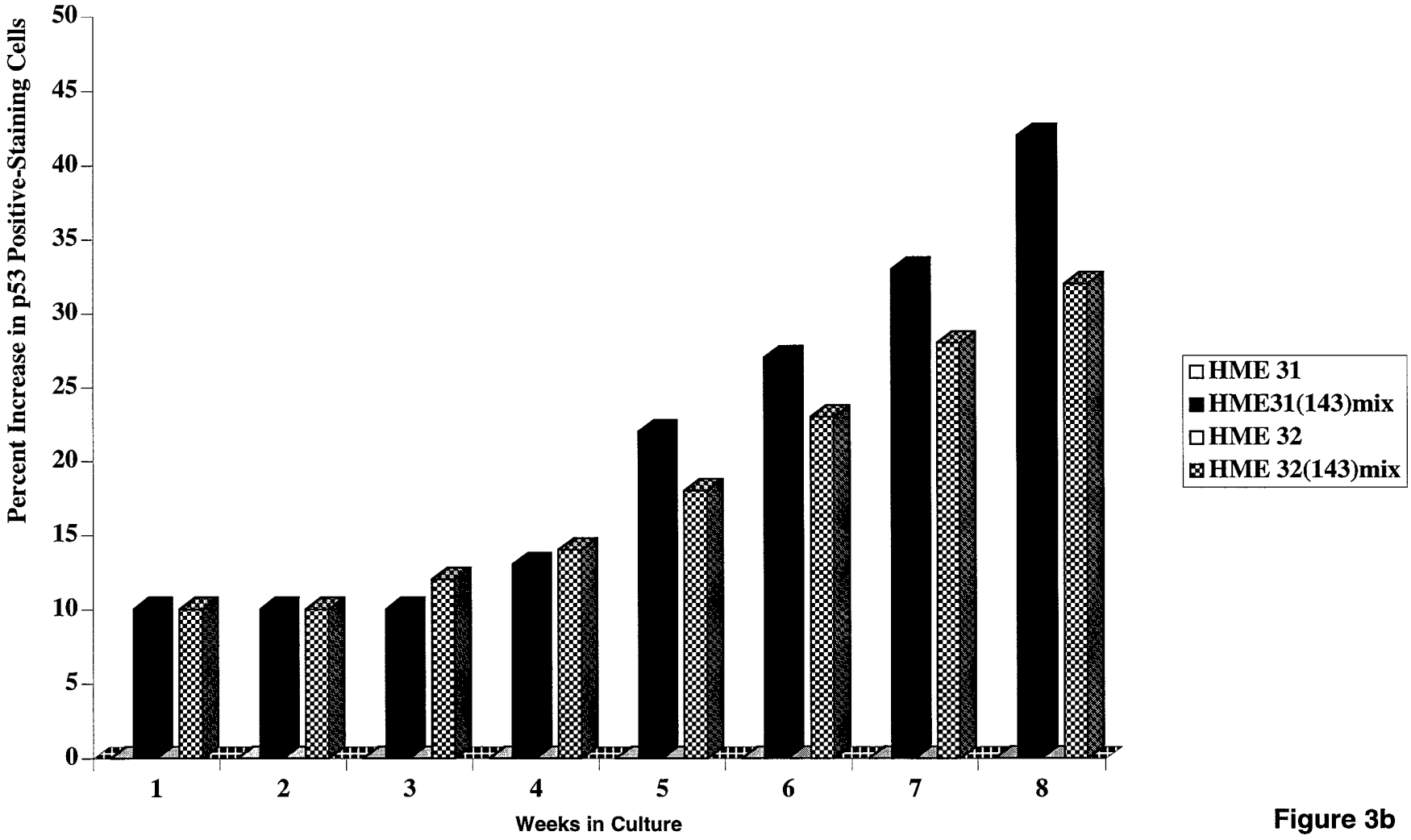

Percent Increase in p53 Positive-Staining Cells
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
Weeks in Culture
HME 31
HME31(143)mix
HME 32
HME 32(143)mix

Figure 3b

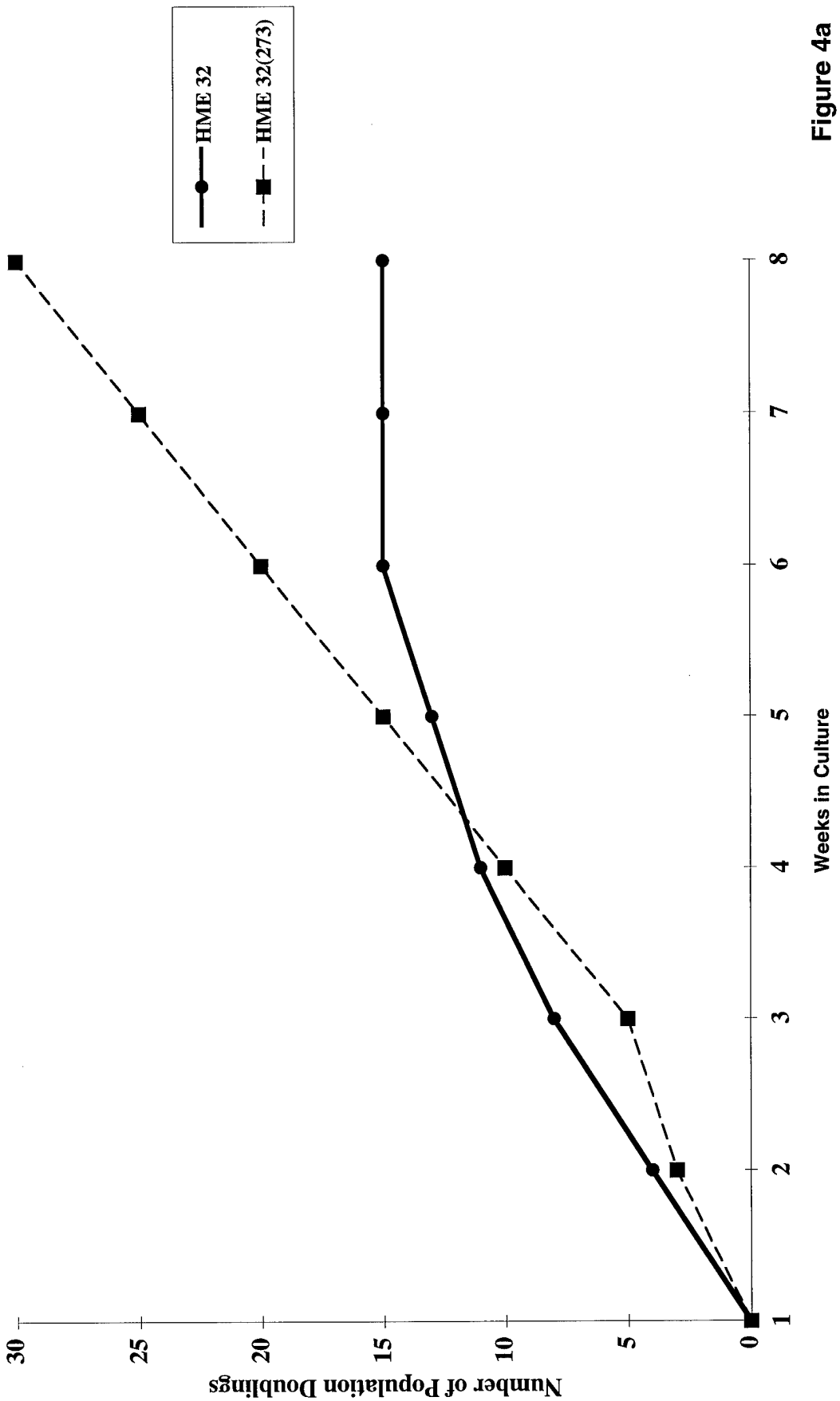

HME 32
HME 32(273)
Number of Population Doublings
0
5
10
15
20
25
30
1
2
3
4
5
6
7
8
Weeks in Culture

Figure 4a

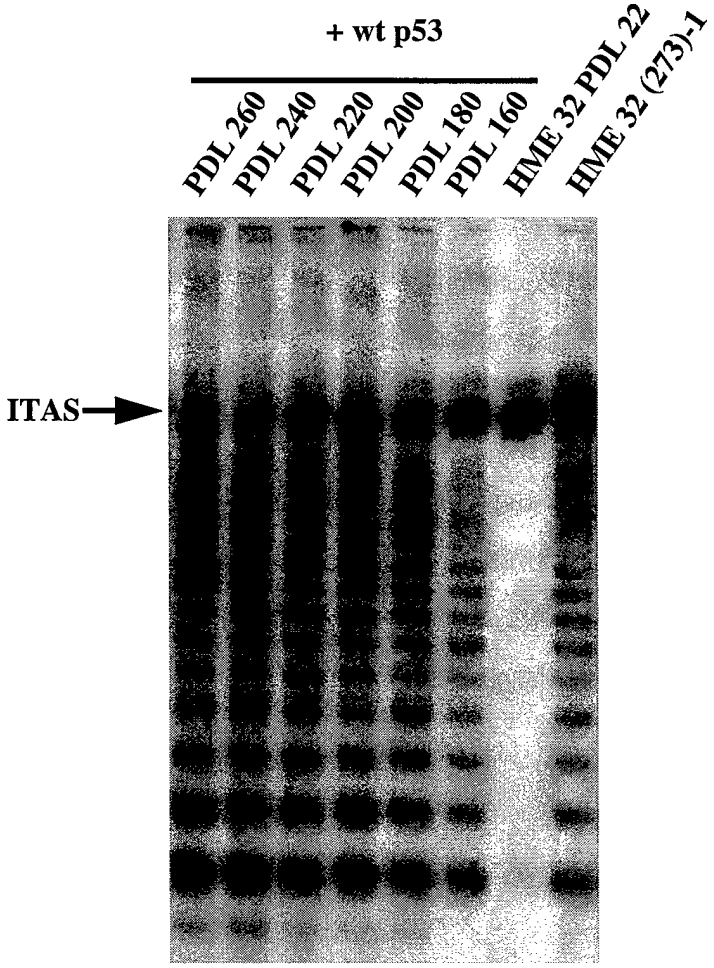

+ wt p53
PDL 260
PDL 240
PDL 220
PDL 200
PDL 180
PDL 160
HME 32 PDL 22
HME 32 (273)-1
ITAS

Figure 4b

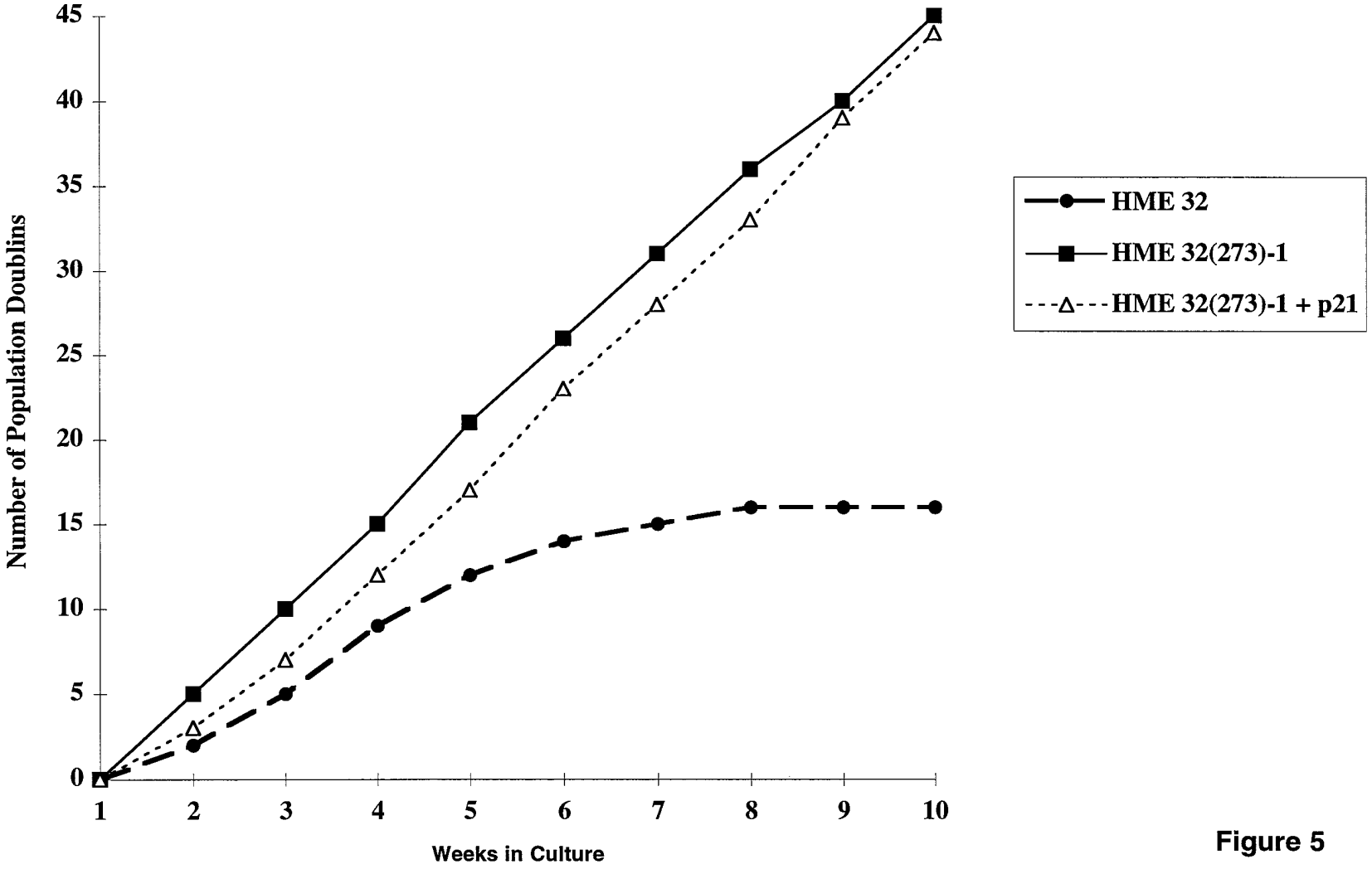
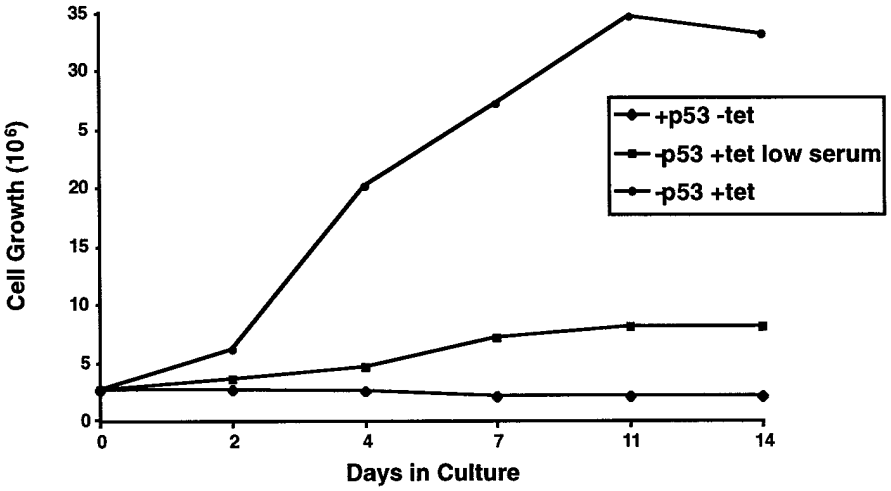

45
40
35
30
25
20
15
10
5
0
Number of Population Doublins
1
2
3
4
5
6
7
8
9
10
Weeks in Culture
HME 32
HME 32(273)-1
HME 32(273)-1 + p21

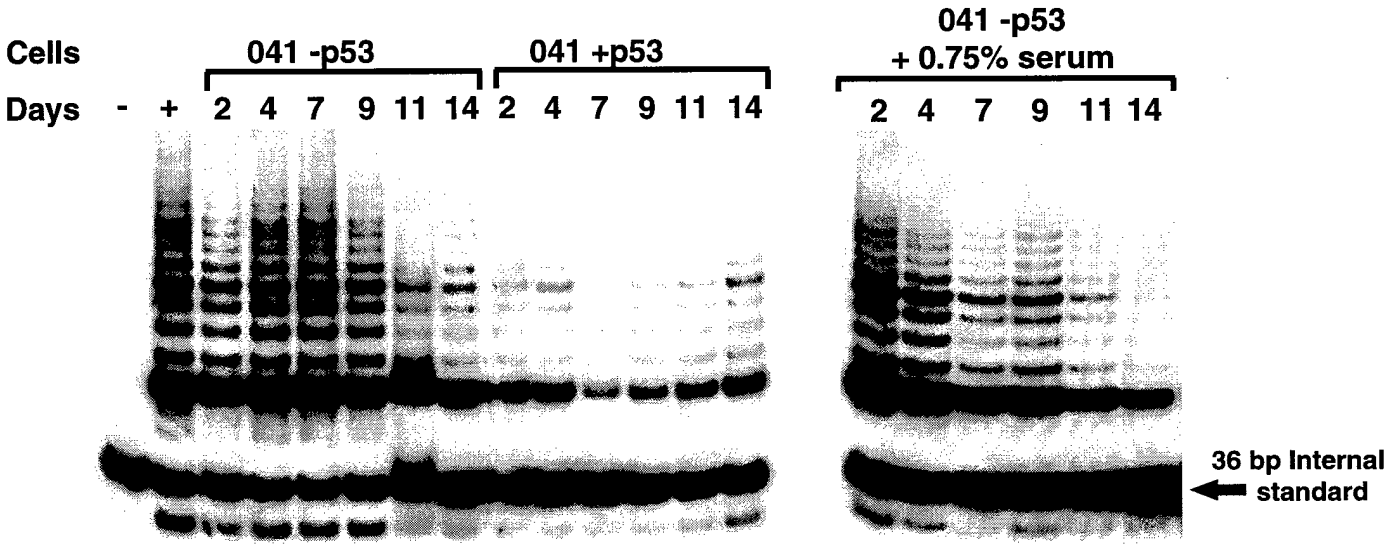
Figure 5

Figure 6

A


Cell Growth (10^6)
35
30
5
20
15
10
5
0
0
2
4
7
11
14
Days in Culture
+p53 -tet
-p53 +tet low serum
-p53 +tet

B


Cells
041 -p53
041 +p53
041 -p53
+ 0.75% serum
Days - + 2 4 7 9 11 14 2 4 7 9 11 14
2 4 7 9 11 14
36 bp Internal
standard

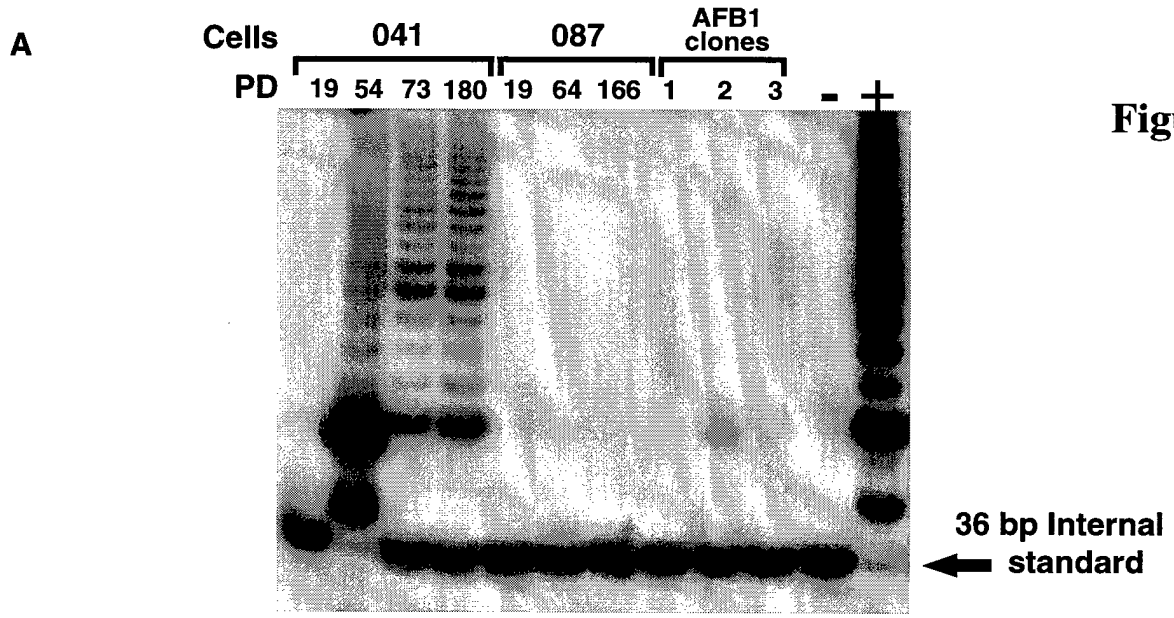

A
Cells
041
087
AFB1 clones
PD
19 54 73 180 19 64 166 1 2 3
-
+
36 bp Internal standard
B
Cells IMR90 1299 087 041
PD 37 NA 46 158 43 56 150 236
p53

Figure 7

Figure 7A

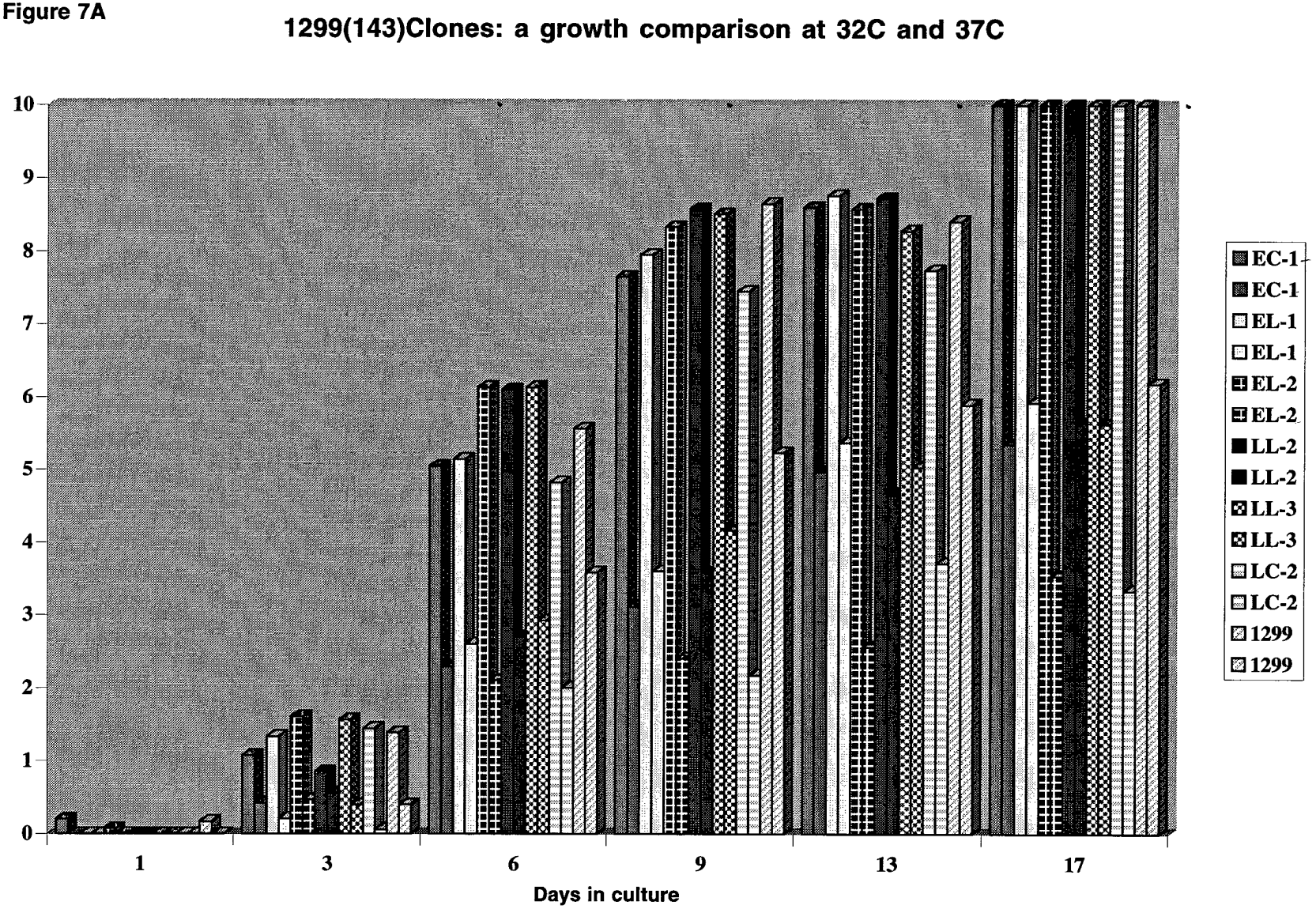

1299(143)Clones: a growth comparison at 32C and 37C
10
9
8
7
6
5
4
3
2
1
0
1
3
6
9
13
17
Days in culture
EC-1
EC-1
EL-1
EL-1
EL-2
EL-2
LL-2
LL-2
LL-3
LL-3
LC-2
LC-2
1299
1299

Figure 8

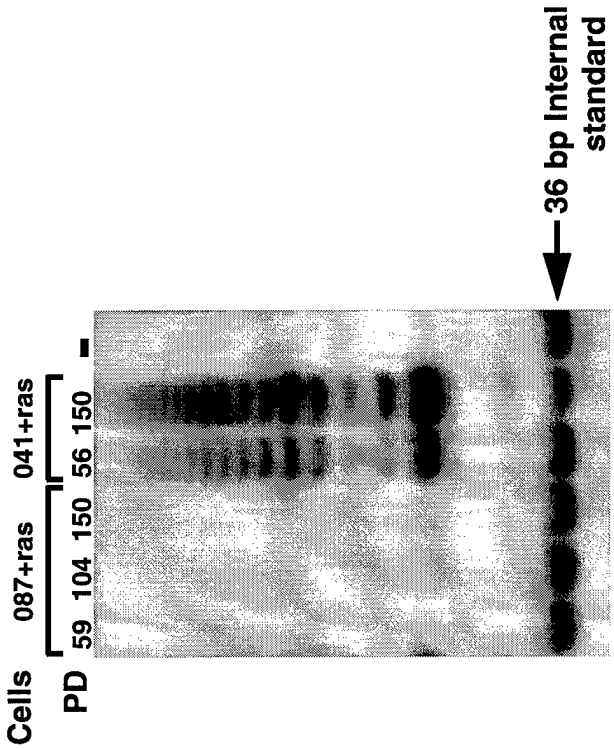
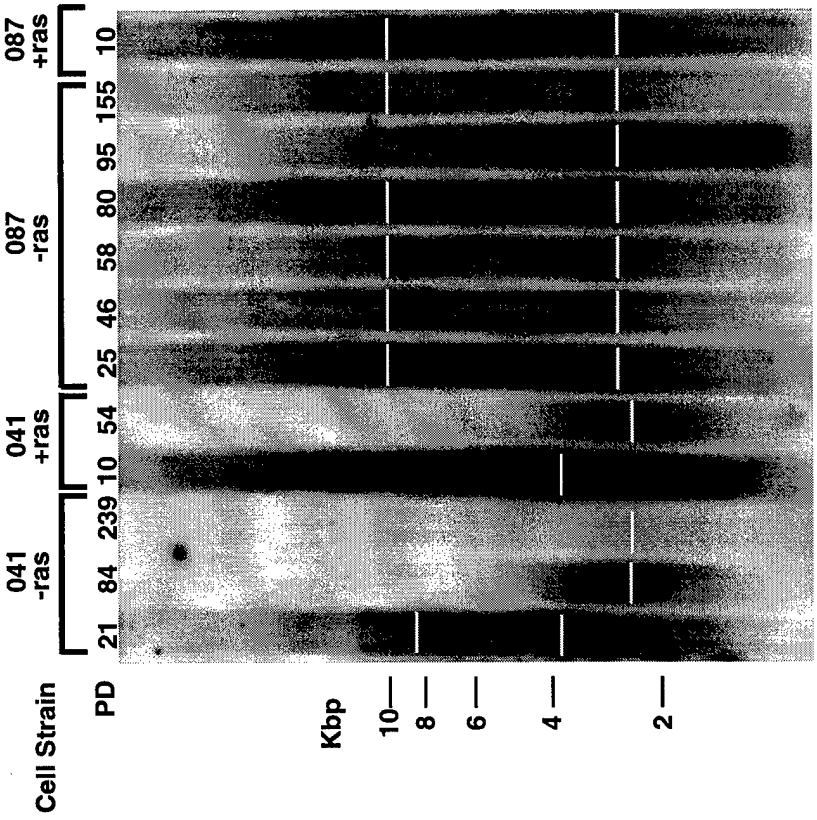

Cells
PD
087+ras
59
104
150
041+ras
56
150
-
36 bp Internal standard

Figure 9


Cell Strain
041 -ras
041 +ras
087 -ras
087 +ras
PD
21
84
239
10
54
25
46
58
80
95
155
10
Kbp
10
8
6
4
2

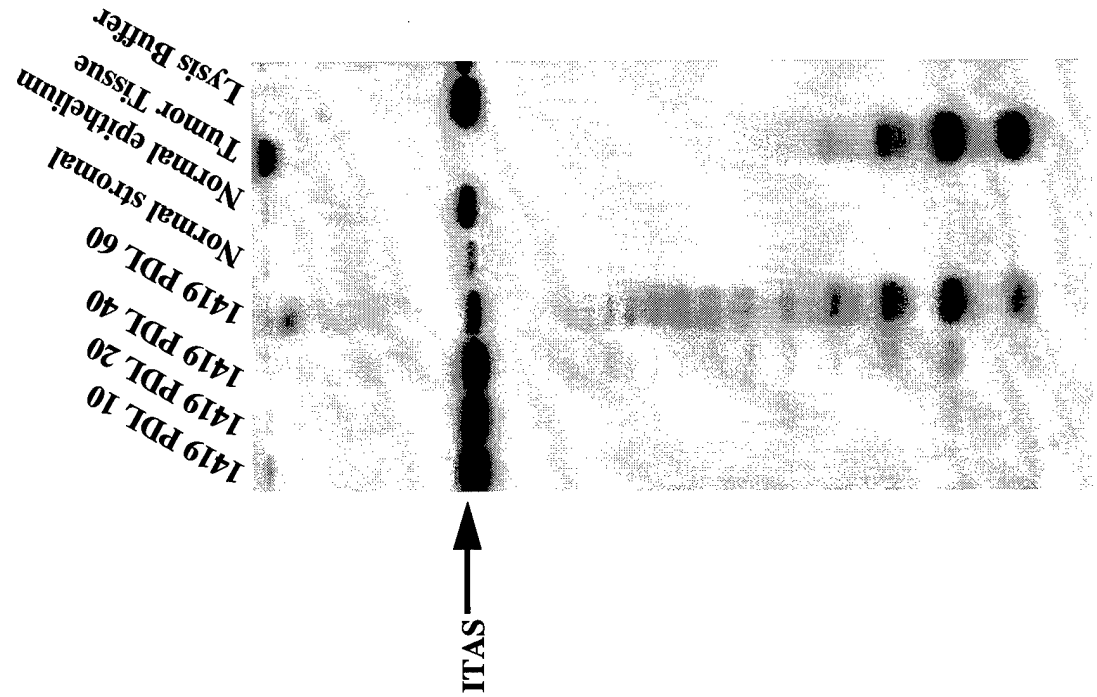

1419 PDL 10
1419 PDL 20
1419 PDL 40
1419 PDL 60
Normal stromal
Normal epithelium
Tumor Tissue
Lysis Buffer
ITAS

Figure 10a

Figure 10b

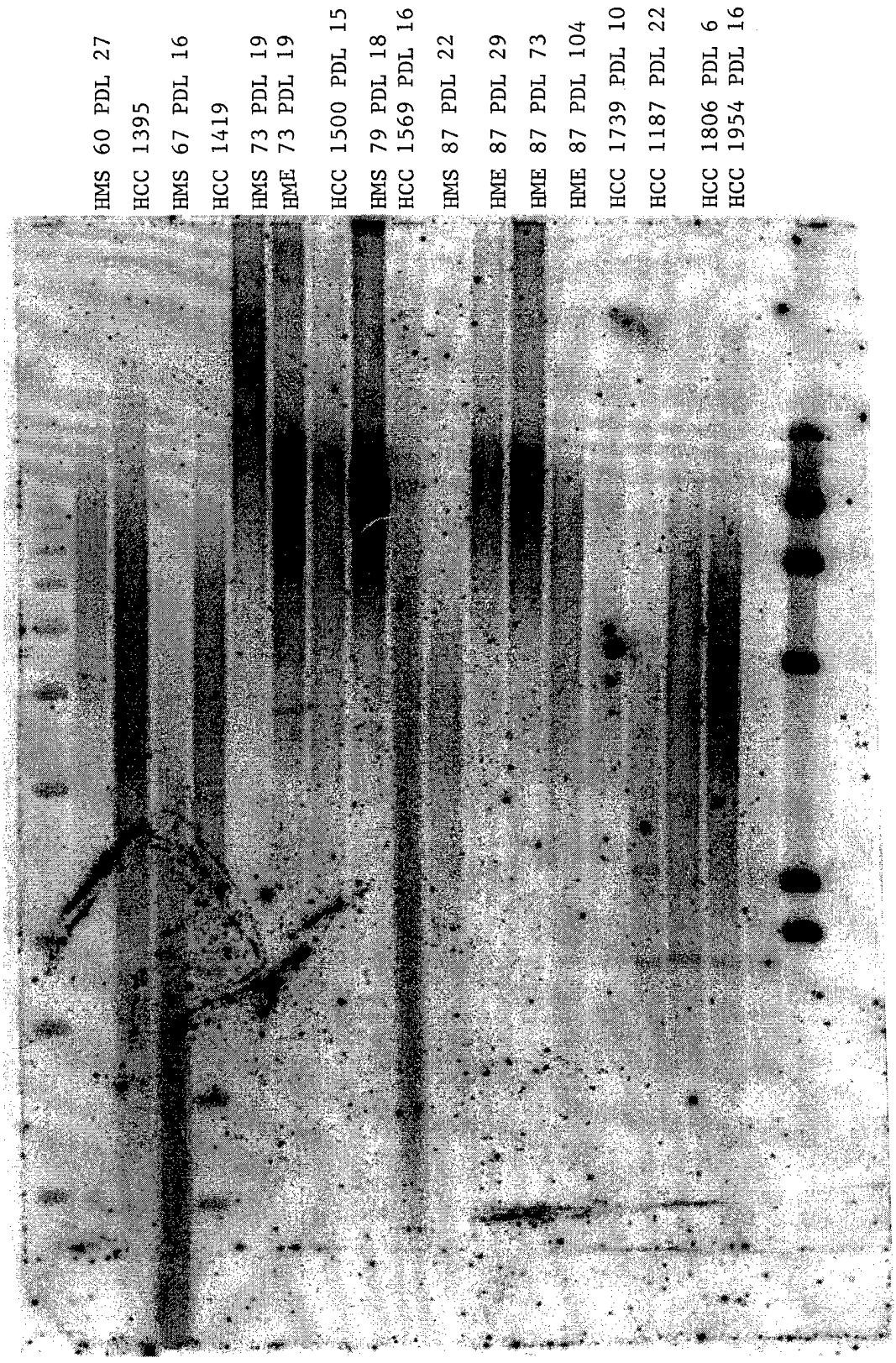

HMS 60 PDL 27
HCC 1395
HMS 67 PDL 16
HCC 1419
HMS 73 PDL 19
HME 73 PDL 19
HCC 1500 PDL 15
HMS 79 PDL 18
HCC 1569 PDL 16
HMS 87 PDL 22
HME 87 PDL 29
HME 87 PDL 73
HME 87 PDL 104
HCC 1739 PDL 10
HCC 1187 PDL 22
HCC 1806 PDL 6
HCC 1954 PDL 16

Figure 11

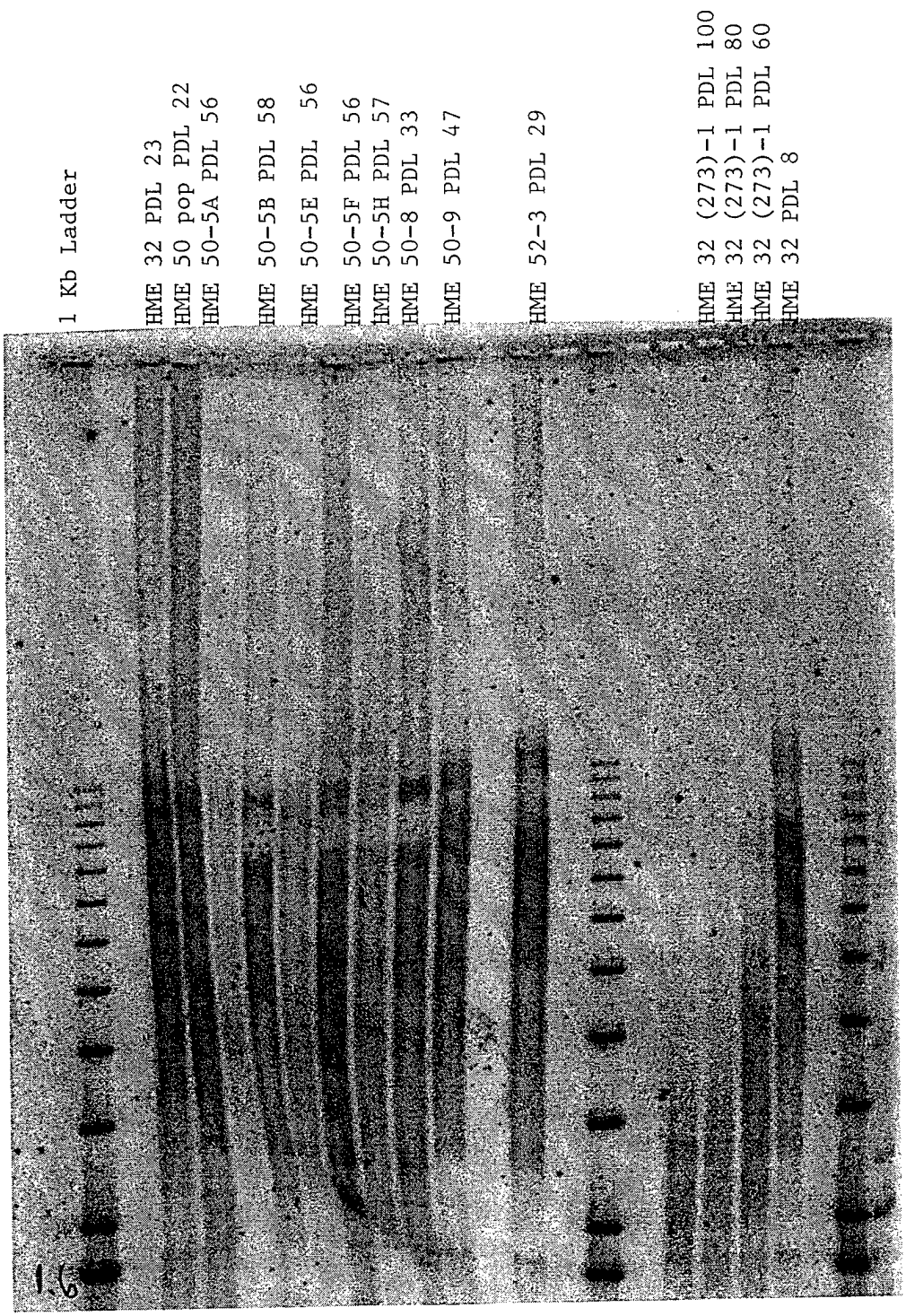

1 Kb Ladder
HME 32 PDL 23
HME 50 pop PDL 22
HME 50-5A PDL 56
HME 50-5B PDL 58
HME 50-5E PDL 56
HME 50-5F PDL 56
HME 50-5H PDL 57
HME 50-8 PDL 33
HME 50-9 PDL 47
HME 52-3 PDL 29
HME 32 (273)-1 PDL 100
HME 32 (273)-1 PDL 80
HME 32 (273)-1 PDL 60
HME 32 PDL 8

Figure 12

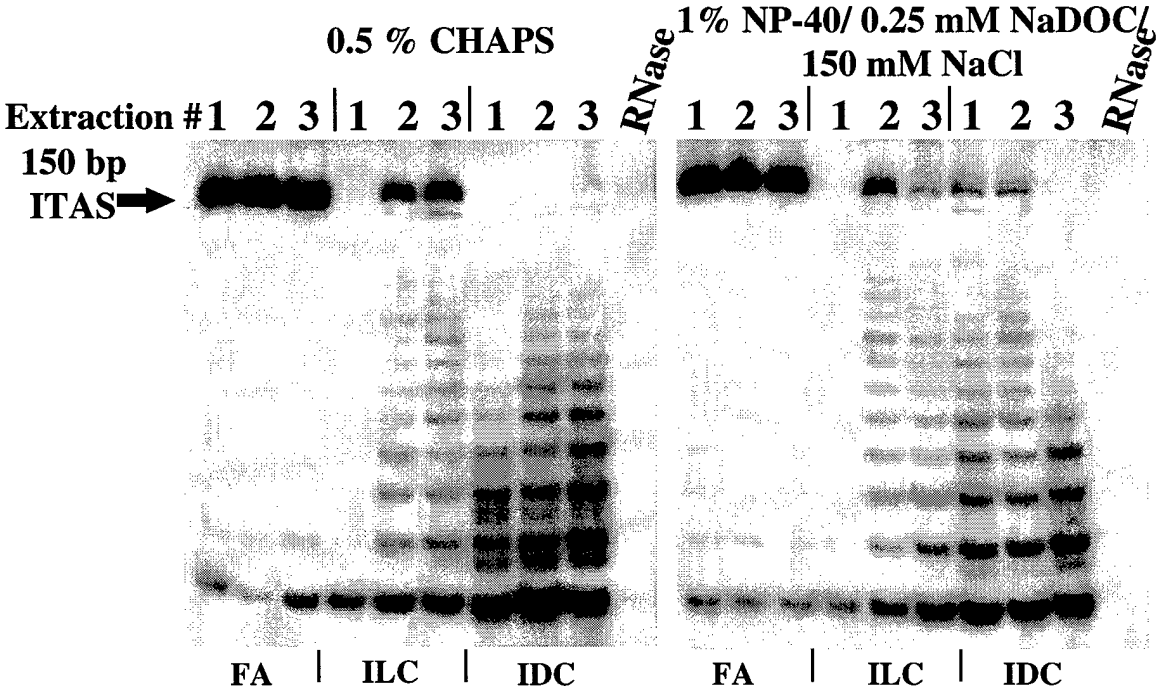

0.5 % CHAPS
RNase
1% NP-40/ 0.25 mM NaDOC/
150 mM NaCl
RNase
Extraction #1 2 3 1 2 3 1 2 3
1 2 3 1 2 3 1 2 3
150 bp
ITAS
FA
ILC
IDC
FA
ILC
IDC

Figure 13a

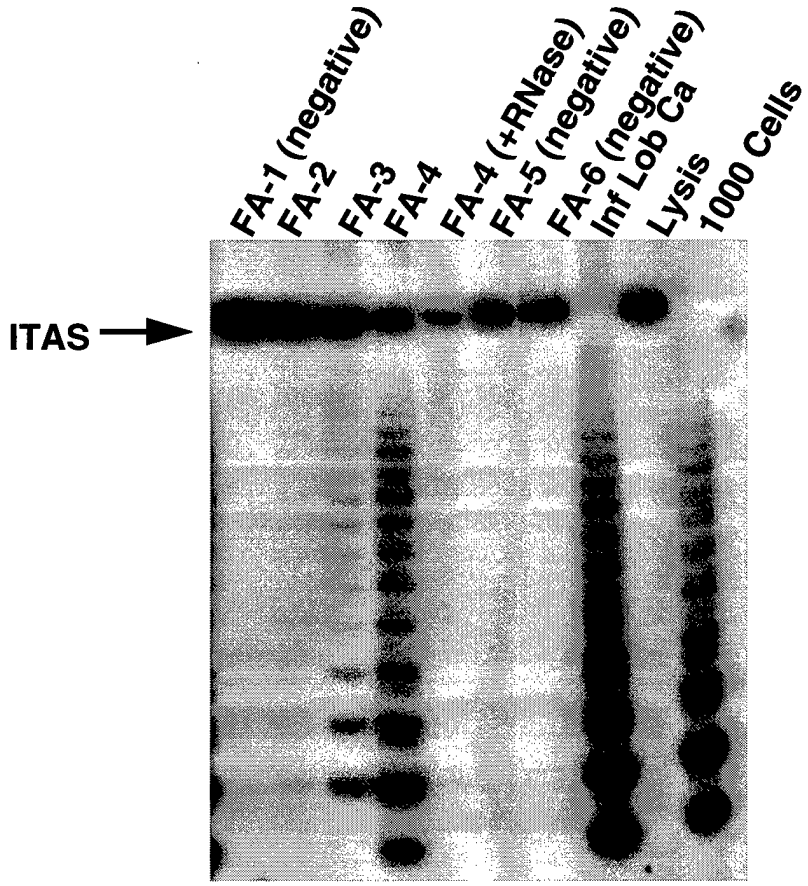

FA-1 (negative)
FA-2
FA-3
FA-4
FA-4 (+RNase)
FA-5 (negative)
FA-6 (negative)
Inf Lob Ca
Lysis
1000 Cells
ITAS

Figure 13b

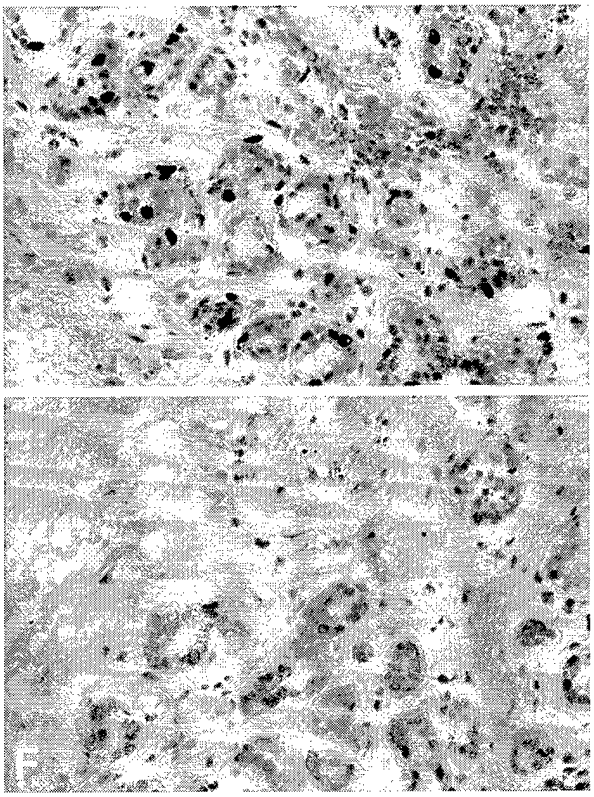


Figure 14

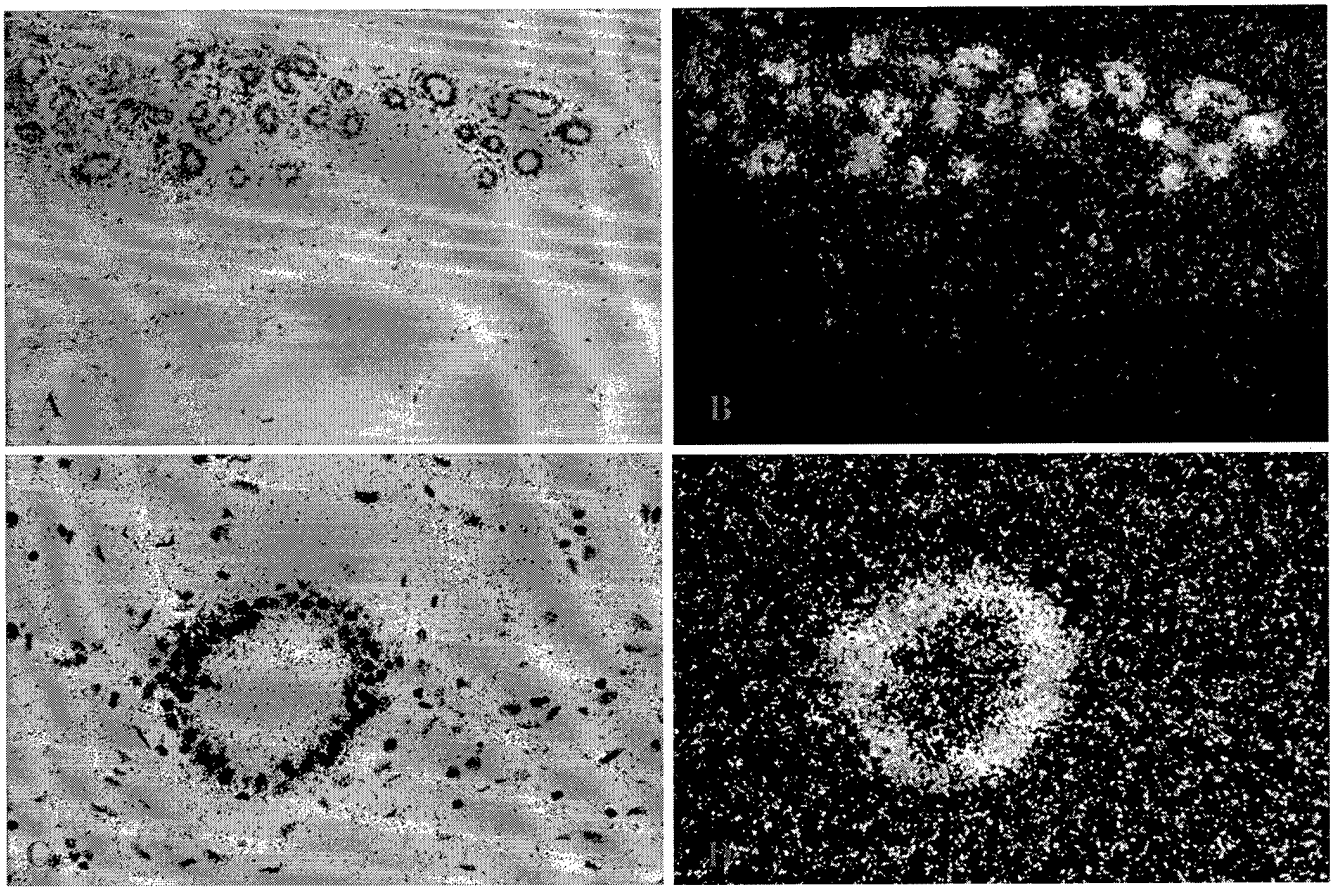
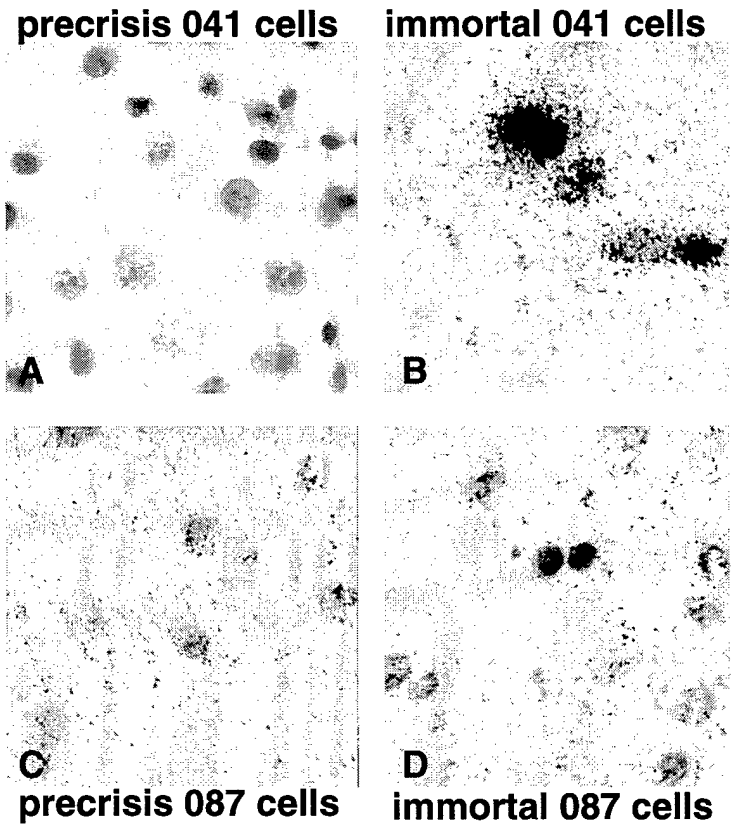


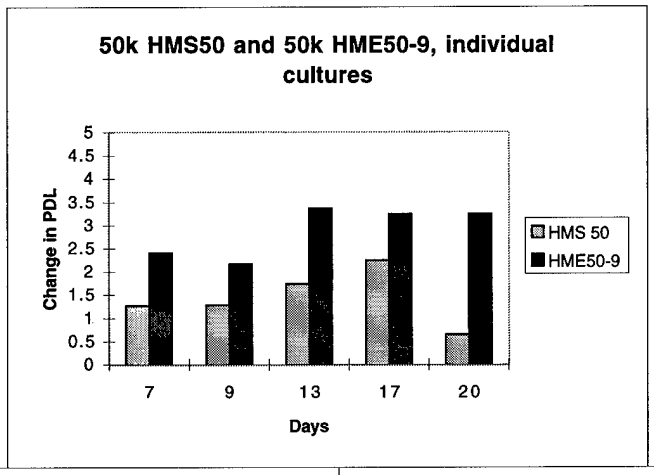
Figure 15

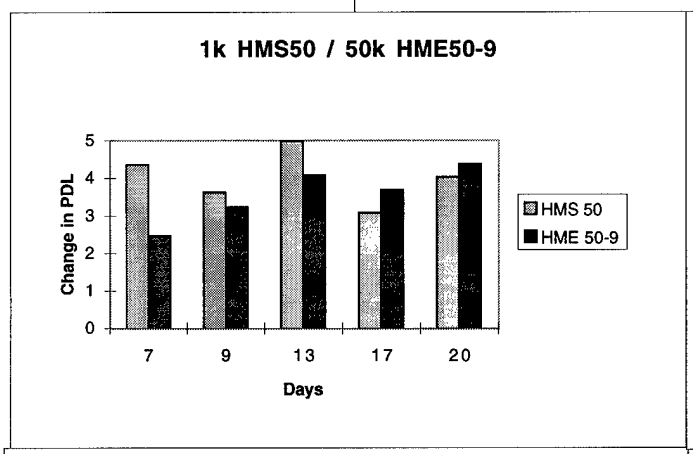

precrisis 041 cells
immortal 041 cells
A
B
C
D
precrisis 087 cells
immortal 087 cells
Antisense hTR

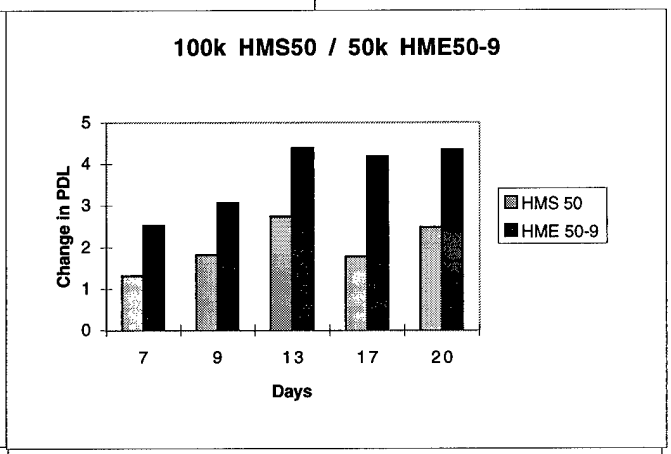
Proliferation Study of HME50-9 in Coculture with HMS50

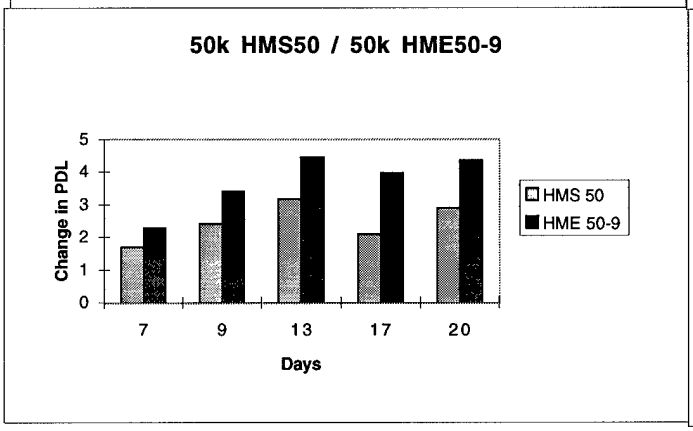
FIGURE 16

In 6-well plates









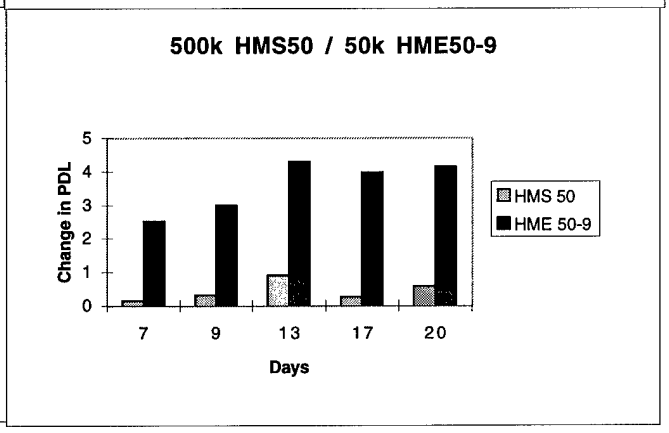
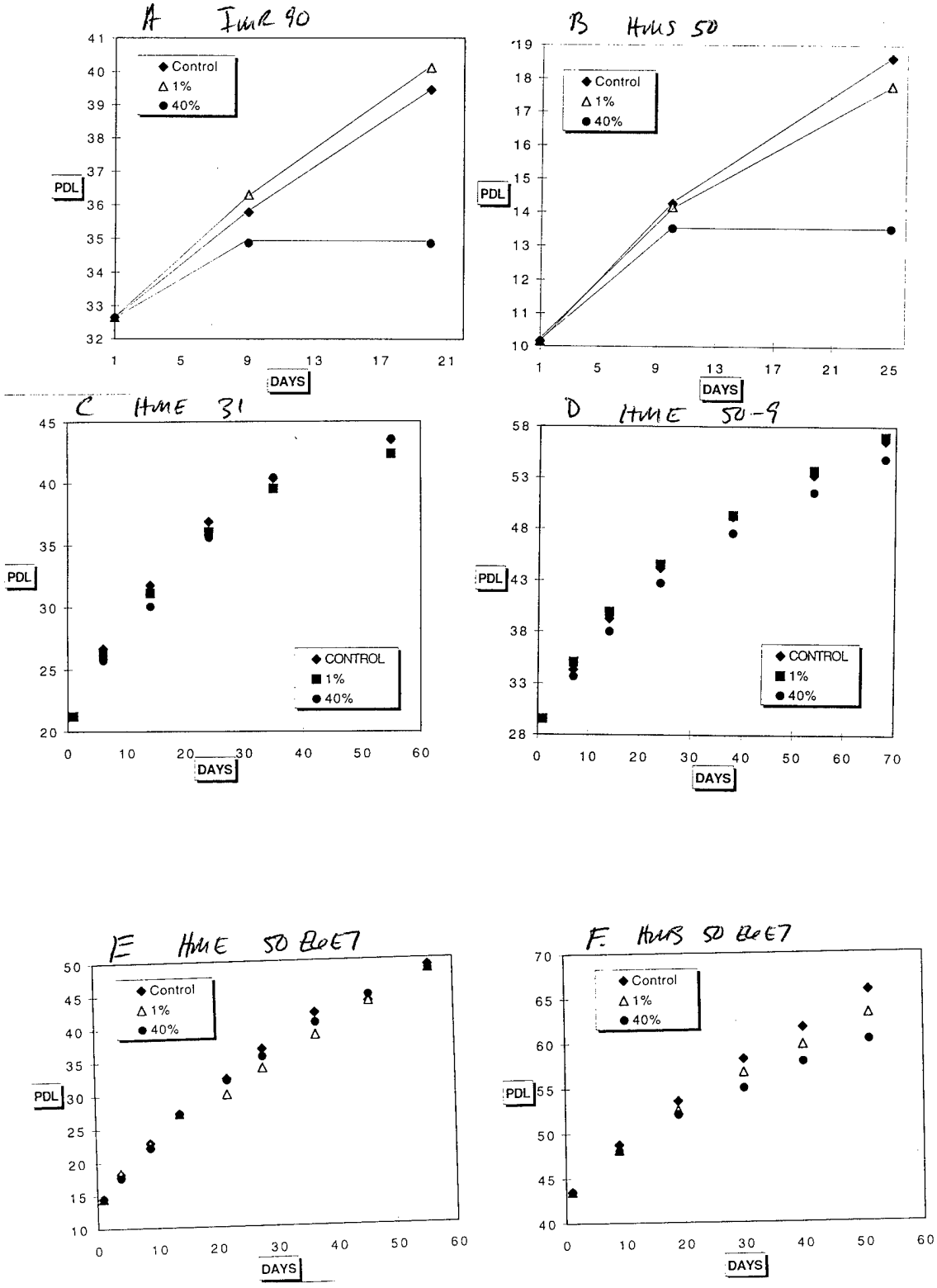


Figure 17


A IMR 90
Control
1%
40%
PDL
DAYS
B HMS 50
Control
1%
40%
PDL
DAYS
C HME 31
CONTROL
1%
40%
PDL
DAYS
D HME 50-9
CONTROL
1%
40%
PDL
DAYS
E HME 50 BoE7
Control
1%
40%
PDL
DAYS
F. HMS 50 BoE7
Control
1%
40%
PDL
DAYS

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Published and Accepted Articles

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